Effects of age, diet, and selected microflora on the large intestinal mucosa of newborn and growing gnotobiotic piglets

Supote Methiyapun
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EFFECTS OF AGE, DIET, AND SELECTED MICROFLORA ON THE LARGE
INTESTINAL MUCOSA OF NEWBORN AND GROWING GNOTOBiotic
PIGLETS

Iowa State University

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Effects of age, diet, and selected microflora on the large intestinal mucosa of newborn and growing gnotobiotic piglets

by

Supote Methiyapun

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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1985
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GENERAL INTRODUCTION

The mammalian large intestine is probably the least studied of all the epithelial structures in the gastrointestinal tract. It is the part of the intestine primarily responsible for the final modification of fluid in the fecal content before it is excreted. The importance of the porcine large intestine is evident when one considers that swine dysentery, a disease that affects only the large intestine, can be a fatal diarrheal disease.

Swine dysentery is a disease caused by an anaerobic spirochete, *Treponema hyodysenteriae*, that affects primarily weaning pigs during the growing and finishing period. The disease has been reproduced with *Treponema hyodysenteriae* in conventional and gnotobiotic piglets with or without selected anaerobic microflora, and it has been suggested that goblet cells may be a primary target cell for the invasion of *Treponema hyodysenteriae*. Large intestinal lesions induced by *Treponema hyodysenteriae* in gnotobiotic pigs are less severe than those induced in the conventional ones. This difference may reflect a dietary effect since gnotobiotic pigs are usually maintained on milk diet, while conventional pigs are maintained on solid food with higher amounts of dietary fibers and protein. In addition, gnotobiotic animals used in swine dysentery experiments are generally younger than conventional ones and the intestine of the former do not contain any bacterial flora. The difference in the severity of the disease between conventional and gnotobiotic pigs may be better understood if the interrelationship between age, diet, and selected bacterial flora is
known. Since sterilized prescription diet (P/D) primarily used for dogs is readily available for use in the gnotobiotic system, it has been utilized as solid food for this experiment. The microflora selected for use in this study included *Selenomonas ruminantium*, *Megasphaera elsdonii*, *Bacteroides sp.*, *Lactobacillus acidophilus*, *Bifidobacterium adolescentis*, and *Acetivibrio ethanolgignens*. They were anaerobic bacteria found in high numbers in the colon of conventional pigs 9 days post experimental *Treponema hyodysenteriae* infection (I. Robinson, NADC, Ames, Iowa, personal communication).

Colonic mucus from the porcine species has been isolated and characterized, but little is known about intracellular goblet cell mucin. Sheehan and Jarvis (1972) investigated and compared the goblet cell mucin histochemistry from various animal species and human. In this study, the porcine species was not included. Morphologically and physiologically, goblet cells from rats, mice, and human have been extensively studied, but goblet cells from pigs have received little attention.

In addition, the development of porcine large intestinal mucosa during postnatal life is not well understood. Wooding et al. (1978) described villus-like structures and vacuolated cells in the large intestine of swine and the possible role of the large intestinal epithelium in fat absorption during the neonatal period. Thus, the cecum and colon of newborn and growing gnotobiotic pigs were evaluated in order to extend our knowledge of the development of this organ and the effects of those factors postulated to impact on the susceptibility
to *Treponema hyodysenteriae*.

The objectives of this study were as follows:

1. To characterize the large intestinal goblet cells in gnotobiotic and/or newborn piglets morphologically, morphometrically, and histochemically.

2. To evaluate the effects of age, diet, and selected microflora on the large intestinal goblet cells of gnotobiotic pigs morphologically, morphometrically, and histochemically.

3. To characterize the large intestinal mucosa of newborn and growing gnotobiotic piglets and its potential in intestinal fat absorption.

**Explanation of Dissertation Format**

This dissertation is presented in the format of the American Journal of Veterinary Research and consists of 3 manuscripts which will be submitted to refereed scientific journals. The first and third manuscripts will be submitted to the American Journal of Veterinary Research. The second manuscript will be submitted to the Anatomical Record.

The general introduction and literature review precede the first manuscript. A summary and discussion follows the last manuscript. A list of references appears at the end of each manuscript. Literature cited in the general introduction, literature review, and summary and discussion appears at the end of the dissertation.
The Ph.D. candidate, Supote Methiyapun, was the principal investigator for each study.
LITERATURE REVIEW

The mammalian large intestine is the part of the gastrointestinal tract primarily responsible for final modification of fluid in the fecal content before it is excreted. Changes in the normal pattern of fluid and electrolyte movement can result in an increase of fecal water excretion or diarrhea and loss of essential ions. The importance of the colon in swine is evident when one considers that swine dysentery, a disease that is limited to the large intestine, can be a fatal diarrheal disease. Therefore, the role of the large intestine may be comparable to that of the renal distal convoluted tubules and collecting ducts.

In addition to water and electrolyte absorption, the large intestine also receives a significant amount of undigested carbohydrates. These carbohydrates are digested by the action of microbial enzymes in the lumen of the large intestine. The end products of microbial carbohydrate digestion are predominantly volatile fatty acids which are utilized as a source of energy by the epithelium or the general body tissues. These volatile fatty acids also facilitate Na and water absorption from the colonic contents. Apart from carbohydrate digestion, large intestinal microbes are also capable of synthesizing vitamin B and microbial protein. Microbial protein may, in turn, be hydrolyzed to yield essential amino acids that can be used by the body.
Porcine Large Intestine

The pig is an omnivorous animal which has a relatively voluminous large intestine suitable for microbial digestion of cellulose and undigested carbohydrates which reach the large intestine in a significant amount each day.\(^6\) Grossly, the large intestine of the pig consists of cecum and colon and is connected by a mesentery to the dorsal abdominal wall between the kidneys.\(^7\) The cecum lies against the dorsal and cranial part of the left flank. Its dorsal end is directly continued by the colon.\(^8\)

The colon consists of three parts: ascending, transverse, and descending colon. The ascending colon is composed of the centripetal and the centrifugal limbs separated by a central flexure, and is arranged in three close, double, spiral coils in the mesentery.\(^9\) Distal to the spiral, the colon passes forward and crosses to the left as transverse colon and then continues caudad as descending colon to the rectum.\(^10\)

The mucosa of the entire large intestine in all domestic species contains ridges or folds on the mucosal surface and generally lacks villi.\(^11\) In newborn pigs, however, structures similar to the small intestinal villi are present until shortly after birth.\(^12\) These villus-like structures are very variable in number and their significance is not yet determined.\(^13\)

The surface of the large intestine is lined by a single layer of tall columnar cells interspersed by high numbers of typical goblet cells. The colonic crypts are straight, tubular structures.\(^14\) At the
base of the crypts, there is a small pool of slowly cycling cells acting as a functional stem for the rest of the crypt cells. With stathmokinetic and labeling techniques, proliferative activity in the crypts has been shown to be relatively low at the base of the crypts, to reach a peak just above it, and then to fall to zero about two thirds of the distance from base to the surface. With progression from base to crypt mouth, cells acquire their functional characteristics and differentiate into columnar absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells. Columnar absorptive and goblet cells make up almost the entire population of cells in the colon.

Undifferentiated crypt epithelial cells

Undifferentiated crypt epithelial cells are recognized by their sparse, short, and irregular microvilli. Their lateral membranes are reasonably straight with multiple desmosomes at their apex. Free ribosomes, polyribosomes, and apical membrane-bound, secretory granules are numerous. These cells are able to proliferate, secrete, and also to differentiate further to cells with other functions, but have little or no absorptive capacity.

Columnar absorptive cells

Columnar absorptive cells in the porcine large intestine have morphological features similar to absorptive cells of other species and to those of the small intestine. They have a microvillus brush border along their apical surface. The area immediately beneath the microvilli, called the terminal web, is relatively free of any
organelles except for microfilaments that extend into the cores of the
microvilli. Mitochondria and rough endoplasmic reticulum are
distributed throughout the cytoplasm. Glycogen granules are frequently
present in massive amounts in the columnar absorptive cells of the pig
colon. The Golgi apparatus is quite small and is located just above
or beside the nucleus. The lateral membrane is consistently folded,
and sealed at the apex by fairly wide tight junctions.

Apart from the typical columnar absorptive cells, colonic mucosa
of newborn pigs also contains epithelial cells with a prominent apical
tubular and vacuolar system (vacuolated epithelial cells). These so
called "vacuolated cells" are found in significant numbers especially
in the area 2-3 cm beyond the ileocecal valve, otherwise their
occurrence is sporadic. When present, these vacuolated cells are
usually located on the protuberances or villus-like structures of the
mucosa. Their physiological importance is not clear. It has been
demonstrated that colostrum injected directly into the colon of newborn
piglets is readily absorbed by these vacuolated cells indicating that
these cells may be involved in colostral absorption. Since these cells
were found in the porcine colon only until shortly after birth, their
role in the colostral absorption has been questioned.

Morphologically, these colonic vacuolated cells in pigs are
similar to vacuolated villous epithelial cells described in the small
intestine of pigs and other species as well as in the rat
colon. It has been shown that columnar absorptive cells of the small
intestine normally differentiate to vacuolated cells at about 4 days
post DNA synthesis in neonatal pigs. Therefore, these vacuolated cells are present in high numbers in the small intestine only during the first 2-3 weeks of life when epithelial replacement is sluggish. After this period, epithelial cell replacement is accelerated, and cells are sloughed off before they reach this stage of vacuolation. Physiologically, the small intestinal vacuolated cells are active pinocytotically. Bovine gamma globulin, ovalbumin, and colostrum are readily taken up by these cells. The decrease and eventual cessation of uptake of macromolecules and colostrum (closure) in rats and mice is associated with the disappearance of these vacuolated cells. In pigs, however, the ileal vacuolated cells are present in 3 week-old pigs long after the cessation of colostrum absorption. These vacuolated cells can take up macromolecular polyvinyl pyrrolidone (PVP) but PVP is not transferred from the epithelial tissue to blood after the animals are five hours old. This indicates that, in pigs, closure more likely results from cessation of molecular expulsion or transport rather than from the loss of vacuolated cells and their ability to absorb macromolecules.

**Goblet cells**

Goblet cells are among the major cell types found in the gastrointestinal epithelium. They are interspersed among columnar absorptive cells and gradually increase in number from the duodenum towards the large intestine. Cheng and Bjerknes (1982) have shown that the number of goblet cells in the mouse intestine increases gradually
from 2.5% of the total epithelial cells in the duodenum to 5.5% in the ileum and 15.8% in the colon. These figures are relatively low with regard to the density of goblet cells seen on histologic sections. This discrepancy is due to their broad shape that creates the false impression that they constitute a majority in the large intestinal epithelium.

**Origin and kinetics** The origin of goblet cells has been a subject of speculation in the literature in the past. As cited by Merzel and Leblond (1969), Bizzozero (1892) noticed transitional cells with granules intermediate between those of Paneth cells and the mucin granules found in goblet cells. He, then, proposed that goblet cells might originate from Paneth cells. Bizzozero's observation was later supported by Hertzog (1937) and Castro et al. (1959). The hypothesis that goblet cells are derived from Paneth cells was challenged by Vialli (1929) and Baecker (1934) who argued that, at least in dogs, cats, and other species which have no Paneth cells, goblet cells must have other precursors.

Transitional cells between goblet cells and argentaffin cells have been observed in the mouse, guinea pig, and man. According to Schofield (1953), goblet cells might originate from argentaffin cells. In contrast, Popoff (1939), interpreted these transitional cells to be a transformation of exhausted goblet cells to argentaffin cells.

In addition to the two hypotheses mentioned earlier, other investigators believed that goblet cells arose from the differentiation
of columnar cells. Chlopkov (1928) described several intermediate stages between the columnar cells and fully differentiated goblet cells. Finally, some investigators believed that goblet cells reproduce themselves by mitosis. Mitosis was described in mucus-containing cells by several workers including Leblond and Messier (1958), Thrasher and Greulich (1966), and Merzel and Leblond (1969).

The controversy about the origin of goblet cells appears to be resolved by use of the radioautographic technique. Chang and Leblond (1971) studied the cell renewal in the descending colon of mice and found that immature absorptive and mucous-containing cells in the lower part of the crypt took up $^{3}$H-thymidine and showed mitotic figures indicating that they are able to proliferate. These observations were later confirmed by Chang and Leblond (1974), and Cheng and Bjerknes (1982). By mathematical calculation, it is evident that mucous cell division accounts for only one-half of mucous cell production in the small intestine and two-thirds of mucous cell production in the descending colon of the mouse. Additional goblet cells are thought to originate from the columnar cell line. These findings suggest that the stem cell pool is not divided into functionally separated groups for each cell type, but that it is composed of multipotential stem cells.

**Morphology** The morphology of goblet cells varies slightly depending on their stage of development. The immature goblet cells, which are also called oligomucous cells by Merzel and Leblond (1969),
are located exclusively in the lower part of the crypt. They are relatively small and contain only a few isolated mucin granules in the supranuclear region. They are capable of taking up $^{3}H$-thymidine and can undergo mitosis as long as their intracellular mucin content is small. As goblet cells migrate from the crypt toward the surface, they become more mature and accumulate a considerable amount of mucin granules in the apical part of the cells. These accumulated mucin granules bulge the cells into the characteristic wine goblet appearance. In general, goblet cells contain an abundant Golgi apparatus in the supranuclear region. Rough endoplasmic reticulum is found in the perinuclear region, often with lamellae paralleling the contour of the nuclear membrane. Mitochondria are found compressed between the nucleus and other elements of the cells in the compressed cytoplasm. Occasionally, mitochondria can be found between mucin granules. The cytoplasm of goblet cells usually has a strong osmiophilic character probably due to compression of free ribosomes and rough endoplasmic reticulum by accumulated mucin granules. The striated border of goblet cells is composed of microvilli with morphology similar to microvilli of columnar absorptive cells but they are generally shorter than those in columnar absorptive cells, are usually irregularly distributed, and have greater electron density.

Occasionally, "pale mucous cells" have been noticed in the small intestinal epithelium of rats and mice. These pale mucous cells are rare and inconsistent. They develop typical goblet shape, but the
cytoplasm and nucleus are pale. The Golgi apparatus is well developed, but the rough endoplasmic reticulum is sparse. The significance of pale mucous cells is unknown.

In addition to the previously mentioned cytoplasmic organelles, the cytoplasm surrounding mucin granules (theca) contains microtubules and intermediate filaments. Microtubules are widely spaced in the theca and are generally vertically oriented. Intermediate filaments are 10 nm in diameter and are arranged into two elaborate systems. One is composed of bundles of intermediate filaments connecting the adjacent desmosomes and running roughly parallel to the lateral plasma membrane. The other appears as a continuous sheet of densely interwoven intermediate filaments that delimits the mass of apical secretory granules. Actin filaments have not been identified in the theca of the goblet cell. The presence of goblet cell cytoskeleton may be involved in the maintenance of cell shape since the characteristic cup-shape of the theca is maintained even after massive exocytosis and deep cavitation of the apical cell surface. Additionally, the microtubules and microfilaments are probably involved in the translocation of mucin granules because drugs that inhibit polymerization of microtubules and actin filaments prevent the normal movement of granules within the goblet.

The shape of goblet cells is, to a large extent, adapted to the thickness of the epithelium to which they belong. In low epithelium, such as the crypts of the intestine, well-developed goblet cells are barrel-shaped with round or piriform theca. They usually attach
broadly to the basement membrane and are often called "unpedunculated" goblet cells.\textsuperscript{55} In the thicker epithelium, the goblet cells assume an ovoid shape. The cytoplasm between the theca and the basement membrane becomes longer and more slender. This type of goblet cells is often called "pedunculated" goblet cell.\textsuperscript{55}

The nucleus of the goblet cell is smaller than that of the other epithelial cells and is generally located in the basal part of the cell.\textsuperscript{55} Its shape varies considerably, but generally it harmonizes with the shape of the cells and the size of the theca. In "unpedunculated" goblet cells, it is usually round or flattened. In "pedunculated" goblet cells, it is ovoid, and oriented longitudinally in the cell.\textsuperscript{55}

**Secretory mechanism of goblet cells**

**Production of mucin** Nassonov (1923) noticed that the first appearance of mucin droplets in the goblet cells took place in the area above the nucleus.\textsuperscript{60} This supranuclear localization of the newly synthesized mucin granules suggested the Golgi apparatus as the site of their formation. Nassonov's findings have been confirmed by several investigators using radioautographic techniques employing \textsuperscript{35}S.\textsuperscript{61-63} From these investigations, it was noted that \textsuperscript{35}S was taken up by the Golgi apparatus and incorporated into the newly synthesized mucin granules indicating that the Golgi apparatus is a site of mucin sulfation.

Kumamoto (1956) observed that after the injection of radioactive glucose, many cells, in particular those secreting mucus, became
radioactive. Subsequent biochemical work by Draper and Kent (1963) indicated that free glucose was incorporated into glycoproteins in the course of their synthesis. By using $^3$H-glucose and $^3$H-galactose, it was shown that both $^3$H-glucose and $^3$H-galactose were used in the synthesis of complex carbohydrate in the Golgi region. Thus, the Golgi apparatus, besides being recognized as the site of sulfation of mucin, is also involved in the synthesis of mucin.

Fate of mucin granules Following release from the Golgi apparatus, mucin granules move into the goblet and are finally discharged. Radioautographically, it was noted that nascent mucin granules did not migrate randomly, but rather moved peripherally along the lateral surface of the goblet from the Golgi apparatus to the apical surface. Administration of drugs that inhibit polymerization of microfilaments and microtubules also inhibit the mucin granule translocation indicating that the movement of mucin granules is under the influence of the microfilaments and microtubules.

Mechanism of mucin release The mechanism of mucin secretion can basically be separated into baseline and accelerated mucin secretion.

Baseline mucin secretion The mucus blanket covering the gastrointestinal mucosa is generally maintained and renewed by the slow, continuous release of mucin from the individual goblet cells throughout the epithelial surface. This slow continuous release of mucin under "baseline" condition is accomplished by conventional exocytosis, involving intermittent fusion of the membrane of a single
mucous secretory granule with the luminal plasma membrane of the cell. Only the mucin granules located at the periphery of the goblet cell are secreted.

The factors that control baseline mucin secretion are unknown. The rate at which mucin granules are transported and released by unstimulated cells has been investigated by radioautographic studies. The time required for the intracellular transit and secretion of mucin granules in individual goblet cells in vivo and/or in organ culture is 4-8 hours in rats, 6-12 hours in rabbits, and 20-24 hours in man. Intraluminal irritants and a variety of substances, for instance, bile acids, cholera toxin, Escherichia coli heat-labile enterotoxin, immune complexes, and neurotransmitters are capable of inducing rapid massive goblet cell release. By using intestinal mucosal explants, potential mediators of rabbit goblet cell secretion have been tested. The direct effect on goblet cells of adrenergic agents, cholinergic agents, intestinal peptide hormones, as well as histamine and serotonin were tested. It was found that only histamine and acetylcholine consistently induced secretory responses in goblet cells and only crypt goblet cells, but not the surface goblet cells, responded. This failure of goblet cells to respond as they moved out of the crypt on to the surface is thought to be related to goblet cell maturation involving a loss or masking of plasma membrane receptors.

The mechanism of accelerated mucin secretion as observed in the intact animal after parasympathetic stimulation includes an exocytosis
of a single apical mucin granule, followed by the orderly, sequential fusion and tandem fission of the closely opposed regions of adjacent mucin granule membranes.\textsuperscript{72} This mechanism of release is called "compound exocytosis" and predominantly involved the granules located in the center of the goblet\textsuperscript{72} which were thought to represent the mucin granules produced earlier in the life of goblet cells.\textsuperscript{73} This process of compound exocytosis, however, is independent of microfilaments or microtubules, the structures involved in the translocation of mucous granules.\textsuperscript{72}

**Gastrointestinal Mucus**

Gastrointestinal mucus is not a single well defined entity.\textsuperscript{84} The term mucus is used loosely to include the viscous slimy fluid of the intestinal lumen, which contains as its major component large glycoproteins (mucins) secreted from goblet cells located in the epithelium.\textsuperscript{84} Other admixtures in mucus include water, electrolytes, sloughed epithelial cells, bacteria and bacterial products, digested food, plasma proteins, bile salts, pancreatic enzymes, and virtually all other constituents normally found in the intestinal juice.\textsuperscript{84}

Mucus forms a viscoelastic gel covering the epithelium throughout the gastrointestinal tract. The mucus secretion can be divided into two components. First, the primary secretion, which is a water insoluble gel adherent to the mucosal surface, and second, the soluble mucus in the lumen.\textsuperscript{85} This soluble mucus in the lumen can come from the adherent mucus gel by proteolytic degradation or mechanical
shearing or can be secreted by the mucosa and passed directly into the lumen.

Functions

The functions of secreted mucus as will be discussed here are based on widely accepted assumptions, such as lubrication and protection of surface epithelial cells. Mostly, the functions of mucus in vivo are not clearly demonstrated.

Lubrication This postulate is consistent with the viscous nature of mucus, which forms a slimy coating over particulate material. It is one of the firmly established assumptions concerning intestinal mucus that it lubricates mucosa, food and fecal material thereby easing the transit of contents along the intestine.

Protection of mucosal surfaces The mucus gel is thought to protect the delicate gastrointestinal mucosa from damage by the mechanical forces associated with digestion and the passage of feces and ingested materials. Heatley (1959) proposed that surface neutralization of acid in the stomach occurred by a mucosal alkaline secretion contained within the adherent mucous gel. Subsequent studies have provided evidence for this hypothesis. In this system, mucous gel provides an unstirred layer and acts primarily as a mixing barrier. The mucosal bicarbonate secretion is confined within the mucus gel matrix and is prevented from rapidly mixing with the much larger amount of acid in the lumen. In addition, mucus has been shown to delay the diffusion of hydrogen ions from lumen towards the epithelium such that neutralization can be achieved.
In addition, gastric mucus is also considered to protect epithelial cells from digestion by pepsin. Mucus is not permeable to molecules in the size of pepsin (MW 35,000) and once pepsin is secreted into the lumen, it will not penetrate through the mucous gel layer to the mucosal surface.

**Interaction with microorganisms** Mucus can provide a barrier to microorganisms and their toxins, as well as parasites. It has been suggested that mucus can physically prevent access of the organism to the mucosal surface. Moreover, mucus may reduce adherence of infectious agents and toxic agents to intestinal epithelium by competitive inhibition of binding to epithelial receptors. In addition, mucus also contains secretory IgA which can bind antigen. Once microorganisms and their toxins are entrapped in the mucus, they are cleaned from the mucosal surface along with fecal materials.

**Providing food for the normal flora** Mucus in the large intestine can provide nutrients for obligate anaerobic bacteria. In the colon, there are subpopulations of anaerobic bacteria which have the complement of glycosidase enzymes that will degrade the carbohydrate component of mucous glycoproteins. Mucus solubilized by proteolytic action in the proximal part of the gut is fully degraded in the large intestine and thus provides a food source for bacteria.
Structure of mucin

The structure of mucin has been extensively reviewed by Hounsell and Feizi (1982)\textsuperscript{101} and Allen (1983).\textsuperscript{102} Briefly, mucins are large molecules with molecular weight ranging from $2 \times 10^5$ to as high as $15 \times 10^6$.\textsuperscript{102} The major portion of the glycoprotein is carbohydrate, which is attached to a central protein core. The average length of carbohydrate chains in a given glycoprotein secretion can vary from 2 to 22 sugars per chain.\textsuperscript{102} N-acetylgalactosamine, N-acetylglucosamine, fucose, galactose, and N-acetyl or N-glycollyl neuraminic acid (sialic acid) are the five major sugar molecules found most commonly in the mucin molecule.\textsuperscript{101} The central protein core is partially buried within the carbohydrate and is resistant to attacks by proteolytic enzymes. As much as 75\% by weight of the glycosylated peptide can consist of threonine, serine, or proline.\textsuperscript{106} Over one-third of the protein core exists as the naked peptide with no carbohydrate side chains and is accessible to proteolysis. The naked peptide contains disulfide bridges that join the glycoprotein subunits together to form the large covalent, polymeric structure of the native glycoprotein.\textsuperscript{106}

Limited information on mucous glycoproteins from human, sheep, pig, and rat indicates that there are wide differences in the molar ratios of the constituent sugars in mucous glycoproteins from various regions of the gastrointestinal tract, as well as in mucous glycoproteins from the same region in different species.\textsuperscript{102} However, N-acetylgalactosamine is always the sugar at the reducing end in an O-glycosidic linkage to serine or threonine residues in the protein.
core. Fucose and sialic acids, when present, are found in the terminal non-reducing positions in the chain.

All gastrointestinal mucous secretions carry a net negative charge due to the presence of substantial amounts of negatively charged ester sulfate and sialic acid residues on the carbohydrate side chains. Sialic acids are always in the terminal positions on the carbohydrate chains while ester sulfate groups are located internally in the chains attached to N-acetylglucosamine. The contents of both sialic acid and ester sulfate vary between species and among molecules within the same secretion.

The sialic acid and ester sulfate content of mucous glycoproteins are the basis for their strong staining with the basophilic dyes, Alcian blue and high iron diamine. The distinction between the sialic acid and ester sulfate is achieved by varying the salt concentration or the pH of the staining solutions or by using neuraminidase to remove the sialic acids. Extensive histochemical studies on colonic mucosa have shown that the degree of sulfation and sialation in the mucous glycoprotein depends on the location of the mucous-producing cells whether they are in the crypts or on the surface, and in which segment of the colon they are located. The amount of mucus secreted and the sulfation, sialation, and sugar composition of its glycoproteins also change in disease states such as carcinoma of the colon and ulcerative colitis in man. A switch from sulfomucins to sialomucins has been observed in the "transitional" mucosa associated with colonic malignancy; this is accompanied by an
increase in the sialic acid content of the isolated glycoprotein.\textsuperscript{105-109} In gnotobiotic piglets experimentally inoculated with \textit{Treponema hyodysenteriae}, there is mucosal hyperplasia followed by a marked loss in sulfomucin from the crypt goblet cells 4 days after inoculation.\textsuperscript{110} The specific function of sulfomucin is not known but it has been suggested that sulfomucin may be related to cellular maturation representing more mature goblet cells.\textsuperscript{111-113} In addition, sialic acid is believed to be important in determining the viscoelastic properties of mucus.\textsuperscript{114}

By using different lectins, it has been shown that various carbohydrate structures in mucin are expressed at different stages of cellular differentiation in human colonic mucosa.\textsuperscript{115} Lectins specific for exposed, non-reducing $N$-acetylgalactosamine residues bind preferentially to mucin synthesized by fully differentiated cells in the upper crypt region. By contrast, lectins specific for exposed galactose residues bind better to mucin produced by immature goblet cells in the lower portion of the colonic crypt.\textsuperscript{115} Additionally, peanut agglutinin, which has a high affinity for the disaccharide structure galactose $^1$-$^3\beta N$-acetylglucosamine, binds to mucin in human colonic cancer\textsuperscript{115} as well as the "cancer-associated" mucin in the histologically normal but potentially premalignant epithelium in the CF-1 mouse.\textsuperscript{116}
Intestinal Microflora

The microflora of the gastrointestinal tract is an enormously complex system consisting of both aerobic and anaerobic microorganisms. It has been estimated that a single individual may harbor more than 400 different bacterial species in the colonic flora.

At birth, the gastrointestinal tract is sterile. Within minutes after birth, the alimentary canal is flooded with microorganisms acquired from the immediate environment. The most important contributor to these microorganisms is the dam.

The lumen of the large intestine of adult swine contains a large population of microorganisms which may exceed $1 \times 10^{11}$ microbes per gm of content. These microorganisms are composed of primarily gram positive and gram negative obligate anaerobic bacteria that are unable to multiply in an atmosphere containing oxygen. Many of the species are intolerent to oxygen and are killed by exposure to it or to growth media or diluting fluids with positive oxidation-reduction potentials. While many of the microorganisms adhere in colonies to surfaces of particles of digesta, such as plant materials, others are associated with epithelial surfaces in the colon and small intestine.

Effects of the microflora on intestinal structure and function

The structural and morphological differences between the intestine of the conventional and gnotobiotic animals emphasized the importance
of an animal interaction with its indigenous microflora. In germ-free rodents, the normal musculature of the cecum fails to develop. Therefore, the cecum is greatly enlarged and is filled with liquid contents. However, the enlargement of the cecum does not occur in non-rodent species such as the germ-free chicken and the germ-free dog. If the intestinal transit time of non-absorbable materials is compared between conventional and gnotobiotic animals, it is evident that substances move through the intestinal tract at a significantly faster rate when a microflora is present. The increased peristaltic activity in the conventional animals is an important host defense mechanism that sweeps pathogenic organisms distally into a more noxious environment.

The overall mass of the small intestine in germ-free animals is decreased, and its surface area is smaller, whereas the villi of the small intestine are uniform in shape and slender. The crypts are shorter and fewer in number than their conventional counterparts. The lamina propria is much thinner in germ-free animals with a reduction in total cellularity.

The epithelial cell turnover rate in germ-free mice and germ-free chickens has been found to be approximately one-half of that of their respective conventional controls. The villus-crypt cell ratios is always higher in germfree mice, rats, chickens, pigs, and dogs than in their conventional counterparts indicating that less proliferating tissue is required to keep the grem-free mucosa intact.
In addition, the microbial flora also stimulates the formation of gastrointestinal lymphatic tissue. The formation of lymphoid nodules takes place very rapidly once the flora begins to establish. Moreover, macrophages from conventional animals have been shown to digest phagocytosed materials more efficiently than macrophages taken from germ-free animals. Macrophage mobilization and subsequent participation in immune-related phenomena have been shown to be increased in animals possessing a microbial flora.

Intestinal Fat Absorption

Fat is an important source of calories and also a solvent for fat-soluble vitamins. In addition, fat is a major structural component of the body especially the membrane of cells and cell organelles. Since fat is insoluble in water, it is necessary for it to dissolve in the watery chyme of the lumen before being absorbed. It is well accepted that fat enters the duodenum from the stomach as unhydrolyzed triglycerides in the form of small droplets which mix with two important secretions: the conjugated bile salts and the pancreatic lipases. The bile salts stabilize the fat emulsion, and lipases hydrolyze triglycerides. About 95% to 100% of ingested triglycerides are absorbed and appear as triglycerides or fatty acids in the circulation. The mechanism of triglycerides absorption from the intestinal lumen was a matter of controversy over the years and the detailed history was reviewed by Cardell et al. (1967) and Marenus and Sjoestrand (1982). Briefly, Palay and Karlin (1959) noted the
small pits containing fat droplets between the microvilli of the small intestine. Additionally, they also observed fat droplets within the cisternae of the endoplasmic reticulum. They proposed that fat was absorbed by pinocytosis into the epithelial cells where the pinocytotic vesicles fused with the endoplasmic reticulum and transferred fat into this system. These fat droplets in the cells, then, were released into the intercellular spaces by reverse pinocytosis.

This hypothesis has been argued as highly improbable since fat would be introduced into the endoplasmic reticulum where sequestration of metabolites and products of cell synthesis normally occur. Additionally, fat absorption by intestinal mucosal cells is not inhibited by lowering the temperature or by metabolic poisoning indicating that it is not an active process. Finally, it has been shown that enzymes necessary for triglyceride synthesis are present in the microsomal fraction of the intestinal cells suggesting that triglycerides of the intestinal epithelial cells are resynthesized from free fatty acids and monoglycerides rather than taken up as unhydrolyzed droplets.

Subsequent studies on the physicochemical nature of lipids in the gut lumen indicated that fat absorption is accomplished by diffusion of monoglycerides and fatty acids rather than triglycerides. Hofmann and Borgstroem (1962) analyzed the gut content during fat absorption; after centrifugation, they found two distinct phases: an oily phase consisting primarily of di- and triglycerides and a micellar
phase made up of free fatty acids, monoglycerides, and conjugated bile salts. They suggested that it is the micellar phase that comes into contact with the microvillous membrane from which the fatty acids and monoglycerides diffuse through the cell membrane. After diffusing into the epithelial cells, fatty acids and monoglycerides are reassembled into triglycerides. The site of resynthesis of triglycerides in the columnar cells, however, is still questionable. Cardell et al. (1967) suggested that synthesis of triglycerides occurs at the membranes of smooth endoplasmic reticulum where chylomicron formation is also completed. After formation, chylomicrons are transported directly to the intercellular spaces or to the Golgi apparatus. Marenus and Sjoestrand (1982), however, demonstrated that the Golgi apparatus is the only organelle in which fat accumulates during early stages of fat absorption. Fat in the apical vesicles, which are predominantly smooth membrane vesicles with no connection with the endoplasmic reticulum, is found in the later stage of fat absorption. They, then, concluded that the Golgi apparatus is primarily involved in the accumulation of triglycerides during fat absorption and that apical vesicles are secondary sites for fat accumulation.

In addition to fat droplets in the Golgi apparatus and in the apical vesicles as mentioned earlier, the large lipid droplets formed in the cytoplasm are commonly observed in the jejunum during advanced absorptive stage as well as in the terminal ileum. They are also observed in the colon of the neonatal piglets. Jersild and Clayton (1971) thought that the presence of these fat droplets in the cytoplasm
of the columnar cells of the terminal ileum is related to the known reduced rate of triglyceride synthesis in this region.\textsuperscript{152} The rate of triglyceride synthesis in the colon of neonatal piglets is unknown.

By using different concentration of fat, Marenus and Sjoestrand (1982) demonstrated that after a meal containing 1.4\% of fat, the Golgi apparatus was the only site at which fat accumulated.\textsuperscript{153} However, if the animals were given meals containing 7-14\% fat, fat was present as large masses free in the cytoplasm in addition to the accumulation in the Golgi apparatus, the apical vesicles, and the endoplasmic reticulum. Later, the number of fat-containing vesicles decreased but the amount of fat in the large vacuoles and free in the cytoplasm increased.\textsuperscript{153} They interpreted this phenomenon to be an abnormal fat absorption caused by a non-physiological flooding of cells with fatty acids and monoglycerides.\textsuperscript{153} In other words, the absorbed amount of fatty acids and monoglycerides exceeded the rate of triglyceride synthesis resulting in flooding of the cells. This is in agreement with the hypothesis proposed by Jersild and Clayton (1971)\textsuperscript{152} as discussed earlier.
SECTION I. EFFECTS OF AGE, DIET, AND SELECTED MICROFLORA ON THE LARGE INTESTINAL GOBLET CELLS OF NEWBORN AND GROWING GNOTOBiotic PIGLETS: HISTOCHEMICAL AND MORPHOMETRIC EVALUATIONS
EFFECTS OF AGE, DIET, AND SELECTED MICROFLORA ON THE LARGE INTESTINAL GOBLET CELLS OF NEWBORN AND GROWING GNOTOBIOTIC PIGLETS: HISTOCHEMICAL AND MORPHOMETRIC EVALUATIONS


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SUMMARY

The effects of age, diet, and selected microflora on the goblet cells of the cecum, proximal, and spiral colon of newborn unfed and gnotobiotic piglets aged 4 to 54 days were evaluated by histochemical and morphometric techniques. Gnotobiotic piglets were divided into 3 groups: one group was maintained on milk, one was on solid food, and one was maintained on solid food and inoculated with 6 strains of microflora selected because they had been found in high numbers in pigs with swine dysentery. The degree of sulfation of goblet cell mucin in milk-fed piglets decreased with age at all three locations in the cecum and in the lower and upper parts of the crypts in the proximal colon. There were neither significant effects of diet nor microflora on the degree of sulfation. The cecum had less sulfomucin than the proximal and spiral colon. The lower part of the crypts had less sulfomucin than the upper part of the crypts and the surface. The volume fraction of goblet cell mucin per unit volume of the large intestinal epithelium ($V_v$) decreased temporarily after the introduction of solid food and selected microflora, but gradually increased again thereafter. The proximal colon had higher $V_v$ than the cecum and spiral colon. The upper part of the crypts had lower $V_v$ than the lower and mid part of the crypts.

The histochemical findings suggested that the large intestinal goblet cells were renewed at a higher rate as the animals get older, and the rate of goblet cell renewal was not dependent on the introduction of solid food or selected microflora.
INTRODUCTION

Goblet cells are one of the major cell components of the gastrointestinal tract. They are interspersed among absorptive cells, gradually increase in number towards the large intestine, and are present in high numbers in the large intestine. Goblet cells produce mucin which provides a protective and lubricant coating over the intestinal mucosa, preventing damage from mechanical and chemical agents, bacteria, viruses, and parasites. The integrity and quantity of the mucus gel may become deranged in a number of diseases, including chronic inflammatory bowel diseases.

Goblet cells may be a primary target cell for Treponema hyodysenteriae, an anaerobic spirochete which causes swine dysentery in pigs during the growing-finishing period. After infection, there is a marked loss in sulfomucin from crypt goblet cells. Although the disease has been reproduced in gnotobiotic pigs, with or without selected colonic microflora, the large intestinal lesions in the gnotobiotic pigs were not as severe as those in the conventional ones. Since gnotobiotic and conventional piglets used in swine dysentery research are different in several aspects including age, diet, and the presence of microflora, we investigated the effects of some of those factors on the large intestinal goblet cells in order to extend our knowledge on their impacts on the pathogenesis of the disease.

The objectives of this study were as follows: first, to evaluate the normal distribution of sialomucin- and sulfomucin-producing goblet
cells in the large intestine of newborn unfed piglets. Second, to evaluate the effects of age, diet, and selected microflora on the distribution of sialomucin- and sulfomucin-producing goblet cells in gnotobiotic piglets, and third, to evaluate the effects of the aforementioned factors on the relative volume of the intracellular mucin content of goblet cells.
MATERIALS AND METHODS

Animals

Five newborn unfed and thirty-eight gnotobiotic piglets were used in this study. They were derived by caesarean section on day 114 of gestation. The newborn piglets were euthanatized right after birth. The gnotobiotic piglets were maintained in rigid tub isolators with a flexible plastic canopy\textsuperscript{13} and were fed evaporated milk with vitamin D added\textsuperscript{a} ad libitum. Four piglets each were euthanatized on day 4 and 14. The remaining thirty piglets were divided into 3 groups of ten pigs each. The first group was maintained on milk diet while the second and third group were given solid food as a preweaning diet in addition to evaporated milk starting on day 14 and were completely weaned to solid food on day 28. The solid food was a sterilized prescription diet (P/D)\textsuperscript{b} containing 8.5\% minimum of crude protein, 5\% minimum of crude fat, and 1\% maximum of crude fiber normally used for dogs. On day 17, each piglet in the third group was given 30 ml per os of an inoculum consisting of 5 ml each of overnight broth cultures of selected microflora, i.e., \textit{Selenomonas ruminantium}, \textit{Megasphera elsdenii}, \textit{Bacteroides sp.}, \textit{Lactobacillus acidophilus}, \textit{Bifidobacterium adolescentis}, and \textit{Acetivibrio ethanoligens}. These organisms had been found in high numbers in the colon of conventional pigs 9 days post

\textsuperscript{a}Carnation Company, Los Angeles, CA.

\textsuperscript{b}Hill's Division, Riviana Foods, KS.
experimental inoculation with *Treponema hyodysenteriae* (I. Robinson, NADC, Ames, Iowa, unpublished data). Four, two, two, and two piglets from each group were euthanatized on day 28, 47, 50, and 54, respectively.

**Tissue Collection and Processing**

Before being sacrificed, the animals were anesthesized with intravenous pentobarbital sodium injection. Tissues from the cecum, proximal colon (2–3 cm distal to the ileocecal valve), and spiral colon (around the central flexure) were collected and were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin blocks. Adjacent tissues saved for future electron microscopic study were fixed in 3% glutaraldehyde in cacodylate buffer. Only formalin-fixed tissues were used in this study.

For histochemistry, sections 5 micrometers thick were cut and stained with high iron diamine-Alcian blue, pH 2.5 (HID-AB).¹⁴ The HID sequence distinguishes HID-positive (black) sulfated acid mucosubstances (sulfomucins) from non-sulfated AB-positive (blue) acid mucosubstances (sialomucins). The degree of HID-positivity (black) was scored in three locations, i.e., the lower and upper part of the crypts and the surface, on the basis of 0 to 4. In this experiment, 0 indicates no HID staining while 4 indicates maximum HID staining.

For morphometric analysis, tissue sections 2 micrometers thick from the antimesenteric side from piglets aged 14 days and older were cut and stained with periodic acid-Schiff (PAS) and counterstained
with Weigert's iron hematoxylin or Mayer's hematoxylin and fast green. Volume fraction of the intracellular mucin content (PAS-positive material) per unit volume of the large intestinal epithelium \( V_v \) was analyzed in three locations, that is the lower, mid, and upper parts of the crypts, by standard point count\(^\text{15} \) using 10 X 10 per mm\(^2 \) ocular grid under oil immersion. Five randomly selected cross sections of crypts in each corresponding location were evaluated.

The \( V_v \) was calculated from the following formula:

\[
V_v = \frac{\text{No. of points falling over PAS positive material} \times 100}{\text{No. of points falling over the large intestinal epithelium}}
\]
RESULTS

Histochemistry

In most of the animals studied, there was a gradual increase in the HID positivity of the goblet cells from the bottom of the crypts to the surface. The goblet cells in the transitional zone almost always had both the HID- and AB-positive materials in the same cell. The HID-positive material was present in the perinuclear region while AB-positive material was in the apical part of the goblet cells close to the lumen.

In newborn unfed piglets, the goblet cells on the surface and in the upper part of the crypts at all segments of the large intestine studied were exclusively HID positive with mean HID scores = 4.0. In the lower part of the crypts, the mean HID scores were 2.8, 3.4, and 3.4 for the cecum, proximal, and spiral colon, respectively (table 1).

The HID scores from milk-fed piglets gradually declined from day 0 to day 54. There were negative correlations between HID scores and the age of the animals at all three locations in the cecum (P < 0.05) (figure 1) and at the lower and upper parts of the crypts in the proximal colon (P < 0.05) (figure 2). In the spiral colon, however, the HID scores also had a tendency to decrease with age in the lower part of the crypts, but this trend was not statistically significant (P > 0.05) (figure 3). The HID scores of the upper part of the crypts of the spiral colon and the surface of the proximal and spiral colon at all ages remained at or close to 4.0, and there was no correlation.
TABLE 1. Effect of age on the HID scores of large intestinal goblet cells of newborn and milk-fed gnotobiotic piglets

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age (days)</th>
<th>No. of animals</th>
<th>Cecum</th>
<th>Proximal colon</th>
<th>Spiral colon</th>
<th>Age means</th>
<th>S.E. of means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DC</td>
<td>SC</td>
<td>SF</td>
<td>DC</td>
<td>SC</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>5</td>
<td>2.8</td>
<td>4.0</td>
<td>4.0</td>
<td>3.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Milk</td>
<td>4</td>
<td>4</td>
<td>2.5</td>
<td>4.0</td>
<td>4.0</td>
<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>4</td>
<td>2.8</td>
<td>3.8</td>
<td>4.0</td>
<td>3.0</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>4</td>
<td>1.8</td>
<td>2.8</td>
<td>3.0</td>
<td>2.5</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>2</td>
<td>2.0</td>
<td>3.0</td>
<td>3.5</td>
<td>2.5</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2</td>
<td>1.5</td>
<td>3.0</td>
<td>3.5</td>
<td>2.0</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>2</td>
<td>1.5</td>
<td>2.5</td>
<td>3.0</td>
<td>2.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

The F-ratio for age = 6.09 (6,16 df; P < 0.05).

\[\text{DC}^b = \text{Deep part of the crypt.}\]
\[\text{SC}^c = \text{Superficial part of the crypt.}\]
\[\text{SF}^d = \text{Surface epithelium.}\]
FIGURE 1. Relationship between HID scores and age in the cecum of newborn unfed and milk-fed piglets (▲ = lower part of the crypt; ● = upper part of the crypt; ○ = surface)
FIGURE 2. Relationship between HID scores and age in the proximal colon of newborn unfed and milk-fed gnotobiotic piglets (△ = lower part of the crypt; ● = upper part of the crypt; ○ = surface)
FIGURE 3. Relationship between HID scores and age in the spiral colon of newborn unfed and milk-fed gnotobiotic piglets (▲ = lower part of the crypt; ○ = upper part of the crypt; O = surface)
between HID scores and age (P > 0.05) (table 1).

There were neither statistically significant differences between the HID scores from milk-fed piglets and the HID scores from P/D-fed piglets with or without anaerobes (P > 0.05) nor interaction between age and treatment (P > 0.05), i.e., the changes in the HID scores with age were the same in all treatments (table 2).

The HID scores were different from one part of the large intestine to the others with the lowest scores in the cecum (2.7) followed by the proximal colon (3.2) and the spiral colon (3.6) (P < 0.05). The scores varied from location to location within each segment of the intestine (P < 0.05) with the lower part of the crypts having the lowest HID scores (2.5) followed by the upper part of the crypts (3.4) and the surface (3.7). There was an additional interaction between the tissues and locations within tissue (P < 0.05), that is the differences in the score among the lower and upper part of the crypts and the surface were not the same in all segments of the large intestine (table 3).

Morphometry

The average volume fraction of the intracellular mucin content per unit volume of the large intestinal epithelium (V_v) in milk-fed piglets increased slightly from 48.6% on day 14 to 50.2% on day 28 and 52.6% on day 47, followed by a decrease to 45.1% on day 50 and a slight increase again to 48.0% on day 54. These changes were not statistically significant different with F ratio = 0.72 (4,9 df;
TABLE 2. Effects of age and treatment on the HID scores of the large intestinal goblet cells

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Treatment</th>
<th>Age means</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Milk</td>
<td>P/D</td>
<td>P/D+Anaerobes</td>
</tr>
<tr>
<td>28</td>
<td>3.2</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>47</td>
<td>3.3</td>
<td>3.1</td>
<td>3.4</td>
</tr>
<tr>
<td>50</td>
<td>3.2</td>
<td>3.2</td>
<td>2.8</td>
</tr>
<tr>
<td>54</td>
<td>2.8</td>
<td>2.8</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Treatment means
(S.E.)
3.1 (0.16)
3.2 (0.16)
3.2 (0.16)

aThe F ratio for treatment = 0.16 (2,18 df; P > 0.05), age = 1.62 (3,18 df; P < 0.05), and age-treatment interaction = 0.33 (6,18 df; P > 0.05).

bMean of 36 measurements for each treatment.

cMean of 18 measurements for each treatment.
TABLE 3. Influence of tissue and location within the tissue on the HID scores of the large intestinal goblet cells

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Location</th>
<th>Tissue means</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower crypt</td>
<td>Upper crypt</td>
<td>Surface</td>
</tr>
<tr>
<td>Cecum&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.8</td>
<td>2.8</td>
<td>3.4</td>
</tr>
<tr>
<td>Proximal colon&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5</td>
<td>3.4</td>
<td>3.8</td>
</tr>
<tr>
<td>Spiral colon&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1</td>
<td>3.9</td>
<td>3.9</td>
</tr>
</tbody>
</table>

| Location means (S.E.) | 2.5 (0.05) | 3.4 (0.05) | 3.7 (0.05) |

<sup>a</sup>The F ratio for tissue = 45.09 (2,36 df; P < 0.05), location = 190.82 (2,108 df; P < 0.05), and tissue-location interaction = 7.33 (4,108 df; P < 0.05).

<sup>b</sup>Mean of 30 measurements for each location.
P > 0.05), and the standard error for age means = 1.18 for age 28 days and 1.66 for the others (table 4). Therefore, these fluctuations were interpreted to be a result of animal variations.

Comparing the $V_v$ from the milk-fed piglets to the ones fed with P/D with or without anaerobic bacterial inoculation (table 4), it was evident that there was an age and treatment interaction ($P < 0.05$) with a marked drop in the $V_v$ to 43.9% for the P/D-fed piglets without anaerobes and to 39.3% for the P/D-fed piglets with anaerobes on day 28. After day 28, the $V_v$ increased gradually in both groups of animals fed with P/D (figure 4).

In addition to the age and age-treatment interaction, the $V_v$ was also influenced by tissues and locations within the tissue. The proximal colon had a higher $V_v$ value (52.1%) than the cecum (46.7%) and the spiral colon (47.4%) ($P < 0.05$). The upper part of the crypts had lower $V_v$ value (44.7%) than the mid (50.0%) and lower parts (51.5%). The difference in the $V_v$ from the lower to mid to upper part of the crypts was the same in all segments of the large intestine ($P > 0.05$) (table 5).
FIGURE 4. Relationship between volume fraction of the intracellular goblet cell mucin per unit volume of the large intestinal epithelium ($V_v$) and age in gnotobiotic piglets.
TABLE 4. Effects of age and treatment on the volume fraction of the intracellular mucin content per unit volume of the large intestinal epithelium (%)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Treatment</th>
<th>Age means</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Milk</td>
<td>P/D</td>
<td>P/D+Anaerobes</td>
</tr>
<tr>
<td>28\textsuperscript{b}</td>
<td>50.2</td>
<td>43.9</td>
<td>39.3</td>
</tr>
<tr>
<td>47\textsuperscript{c}</td>
<td>52.6</td>
<td>51.1</td>
<td>46.7</td>
</tr>
<tr>
<td>50\textsuperscript{c}</td>
<td>45.1</td>
<td>52.3</td>
<td>53.1</td>
</tr>
<tr>
<td>54\textsuperscript{c}</td>
<td>48.0</td>
<td>58.6</td>
<td>56.7</td>
</tr>
</tbody>
</table>

Treatment means (S.E.)

- Treatment means:
- Milk: 49.2 (1.29)
- P/D: 50.0 (1.29)
- P/D+Anaerobes: 47.0 (1.29)

\textsuperscript{a}The F ratio for treatment = 1.44 (2,18 df; P > 0.05), age = 8.73 (3,18 df; P < 0.05), and age-treatment interaction = 4.38 (6,18 df; P < 0.05).

\textsuperscript{b}Mean of 180 measurements for each treatment.

\textsuperscript{c}Mean of 90 measurements for each treatment.
TABLE 5. Influences of tissue and location within the tissue on the volume fraction of the intracellular mucin content per unit volume of the large intestinal epithelium (%)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Location</th>
<th>Tissue means</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower crypt</td>
<td>Mid crypt</td>
<td>Upper crypt</td>
</tr>
<tr>
<td>Cecum^b</td>
<td>50.0</td>
<td>46.6</td>
<td>43.6</td>
</tr>
<tr>
<td>Proximal colon^b</td>
<td>54.7</td>
<td>53.6</td>
<td>47.9</td>
</tr>
<tr>
<td>Spiral colon^b</td>
<td>49.8</td>
<td>49.8</td>
<td>42.6</td>
</tr>
<tr>
<td>Location means</td>
<td>51.5</td>
<td>50.0</td>
<td>44.7</td>
</tr>
<tr>
<td>(S.E.)</td>
<td>(0.70)</td>
<td>(0.70)</td>
<td>(0.70)</td>
</tr>
</tbody>
</table>

^The F ratio for location = 25.91 (2,1188 df; P < 0.05), tissue = 6.88 (2,36 df; P < 0.05), and tissue-location interaction = 0.89 (4,1188 df; P > 0.05).

^bMean of 150 measurements for each location.
DISCUSSION

The results from this experiment indicate that the sialomucin producing goblet cells are located at the bottom of the crypts in all segments of the large intestine studied. There is a gradual increase in sulfomucin content in the goblet cells toward the mucosal surface, and sulfomucin is almost always the only acid mucosubstance present on the luminal surface. It has been suggested that sulfomucin production may be related to cellular maturation representing more mature goblet cells.\textsuperscript{14,16-17} Our findings together with the fact that goblet cells migrate from the bottom of the crypts to the surface where they are extruded,\textsuperscript{18-22} suggest that in pigs sialomucin is produced primarily by immature goblet cells located at the bottom of the crypts while sulfomucin appears to be a product of more mature goblet cells on the large intestinal surface.

Goblet cells in the transitional zone of the crypts contain both sialomucin and sulfomucin in the same cells with sulfomucin located in the supranuclear region, where the Golgi apparatus is located, while sialomucin is present near the apex of crypt goblet cells. Mucin has been shown to be produced and sulfated in the Golgi apparatus of the goblet cells.\textsuperscript{23-25} Therefore, the presence of sulfomucin closer to the Golgi apparatus of the goblet cells in the cell where sialomucin is also present may indicate that along with maturation of goblet cells from crypt to surface, there is a switch from primarily sialomucin production to sulfomucin production. The presence of primarily sulfomucin in the goblet cells at the surface indicates
that they are fully mature goblet cells.

Mucosal hyperplasia followed by the disappearance of sulfomucin from crypt goblet cells has been reported in gnotobiotic piglets experimentally inoculated with *Treponema hyodysenteriae* and it was hypothesized that pathogenic *Treponema* are capable of interacting with the mucus release of goblet cells and that they induce an increased rate of cell renewal at the bases of the crypts. This hypothesis is in agreement with our findings that in pigs, a switch from sialomucin to sulfomucin production is likely related to the age of goblet cells.

Radioautographically, it has been shown that in mature goblet cells, newly synthesized mucin granules migrate from the Golgi region along the periphery of the goblet. These peripherally located mucin granules are the mucin granules secreted under normal "baseline" secretion that involves the intermittent release of single secretory granule. The "accelerated" secretion of mucin evoked by secretagogues, on the other hand, is accomplished by compound exocytosis that involves fusion of multiple granule membranes with the plasma membrane and with each other. Under this accelerated secretion only the centrally-stored granules of the crypt goblet cells, which are thought to represent mucin granules produced early in the life of the goblet cells, responded to the stimuli. It was, therefore, suggested that chemical differences may exist in the mucus released from the goblet cells under baseline vs. accelerated conditions. If we assume that this same phenomenon occurs in pigs, it would be reasonable to suggest that the earlier produced, centrally
located mucin (sialomucin) may be an important mucin component that provides mucosal protection under accelerated stimulations of goblet cell secretion. Sialic acid is believed to be important in determining the viscoelastic properties of mucus, and the highly viscous nature of sialomucin may help to protect the colonic epithelium from damage by foreign materials.

The cecum appears to have more sialomucin producing goblet cells in the lower and upper parts of the crypts than the proximal colon and the spiral colon. The significance of these differences is not known but may reflect a higher rate of goblet cell migration in the cecum of gnotobiotic pigs than in the proximal and the spiral colon. This is quite different from mice and gerbils in which the cecum contains more sulfomucin than sialomucin. Our results are similar to the description in rats, rhesus monkeys, and man which have more sulfomucin than sialomucin in the upper region of the crypts, but are different from guinea pigs, hamsters, cats, and dogs in which sulfomucin is almost exclusively present in both the cecum and proximal colon.

The gnotobiotic system allowed us to evaluate the effects of age, diet, and selected microflora individually on the goblet cell histochemistry and morphometry. From histochemical studies, there is no difference in the HID scores between milk-fed pigs and P/D-fed pigs whether they were fed with or without anaerobes. Therefore, it is interpreted that neither the solid food alone nor in combination with selected microflora influence the degree of mucin sulfation in the large intestine of pigs in the age groups investigated.
Interestingly, the degree of sulfation in the lower and upper parts of the crypts in the cecum and the proximal colon has negative correlation with the age of the animals. We interpret this negative correlation to be due to an age-related increase in goblet cell renewal rate. There is, however, no information on the rate of goblet cell renewal in gnotobiotic piglets available to confirm our interpretation.

Morphometrically, it is evident that the $V_v$ of the intracellular mucin content was markedly reduced in pigs on P/D or P/D with anaerobes as compared to milk-fed pigs, and the effect of P/D and anaerobes appear to be additive. After day 28, the $V_v$ increased gradually until day 54 when the experiment was terminated. We do not know whether this change in the $V_v$ is a result of the change in goblet cell number or the change in the amount of intracellular mucin per goblet cell. We, however, interpret this drop in the $V_v$ to be a response of goblet cells to the introduced solid food and selected microflora. The release of intracellular goblet cell mucin content can be mediated by immune mechanisms,$^{33-34}$ direct irritation,$^{23}$ or parasympathetic stimulation.$^{27}$ A temporary reduction in $V_v$ of goblet cell mucin after the introduction of solid food may be important in the pathogenesis of post-weaning diarrhea in pigs if the same phenomenon occurs in conventional animals. Increased cell production and a reduced rate of cell turnover and hence a decreased rate of cell exfoliation were shown to occur in rats fed certain dietary fibers.$^{35}$ The presence of bacterial flora has also been shown to be associated
with increased cellular proliferation in the ileal epithelium. The increase in $V_v$ after day 28 was interpreted to be an adaptive response to direct irritation from solid food and inoculated anaerobic bacteria.

In conclusion, the degree of goblet cell mucin sulfation in the large intestine decreased with age. Neither solid food alone nor in combination with selected microflora had any significant effects on the degree of sulfation. The findings suggested that the large intestinal goblet cells were renewed at a higher rate as the animals get older, and there were no effects of solid food or selected microflora on the goblet cell renewal rate. Solid food and selected microflora caused marked increase in $V_v$ after a transient reduction. Whether this increase in $V_v$ is due to an increase in the number of goblet cells or an increase in the amount of intracellular mucin per goblet cell needs further evaluation.
REFERENCES


SECTION II. THE LARGE INTESTINAL GOBLET CELLS OF NEWBORN AND GROWING GNOTOBIOTIC PIGLETS: EFFECTS OF DIET AND SELECTED MICROFLORA
THE LARGE INTESTINAL GOBLET CELLS OF NEWBORN AND GROWING
GNOTOBIOTIC PIGLETS: EFFECTS OF DIET AND SELECTED MICROFLORA

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The senior author is the scholarship recipient from the Royal
Thai Government.
SUMMARY

The morphology of goblet cells from the cecum, proximal colon, and spiral colon of newborn and growing gnotobiotic piglets aged 4 to 54 days was evaluated by histology and electron microscopy. The gnotobiotic pigs were divided into 3 groups: one was maintained on milk, one was on solid food, and one was on solid food and inoculated with 6 strains of microflora which had been found in high numbers in pigs with swine dysentery. Goblet cells from the large intestine of newborn and growing gnotobiotic piglets were similar to those from other species, but most of them appeared to have the same level of electron density as other epithelial cells in contrast to the high electron density of goblet cells from other species. Occasionally, pale goblet cells which were interpreted to be a less active form of this cell type were found in the mid crypt epithelium of all three large intestinal sites. Four out of eight milk-fed piglets aged 4 and 14 days and 9 out of 10 milk-fed piglets aged 28 to 54 days had swollen goblet cells in the mid crypt region of the cecum. Only 5 out of 10 piglets on solid food without selected microflora and 9 out of 10 piglets on solid food with selected microflora had these swollen goblet cells in the cecum. Solid food and selected microflora had a minimal effect on the morphology of large intestinal goblet cells.
INTRODUCTION

Goblet cells are one of the major cell types interspersed among the columnar absorptive cells in the intestinal epithelium. Their ratio has been shown to gradually increase from 2.5% of the total epithelial cells in the duodenum to 5.5% in the ileum and 15.8% in the colon of mice. The primary function of goblet cells is to produce and secrete mucins, the high molecular weight glycoproteins responsible for the viscous nature of mucus.

Several porcine enteric diseases, for instance, porcine intestinal adenomatosis (PIA) and swine dysentery affect the large intestine. In PIA, the lesions are primarily in the terminal ileum and proximal part of the large intestine and are characterized by marked mucosal hyperplasia with absence of goblet cells. In swine dysentery, on the other hand, hyperplasia of immature goblet cells appears to be a characteristic feature of the disease. The disease has been reproduced in conventional and gnotobiotic pigs with or without selected colonic microflora, and goblet cells appeared to be a primary target cell for the invasion of the causative agent, Treponema hyodysenteriae. The large intestinal lesions in gnotobiotic pigs were less severe than those in the conventional ones suggesting that several factors including age, diet, and microflora may influence the susceptibility to Treponema hyodysenteriae. In addition, goblet cells from rats and mice have been extensively studied, but those from the pigs have received little attention.
The objectives of this study were: i) to characterize the goblet cell morphology in newborn and growing gnotobiotic piglets, and ii) to evaluate the effect of solid food and selected microflora on goblet cell morphology.
MATERIALS AND METHODS

Animals

Five newborn unfed and thirty-eight gnotobiotic piglets were used in this study. They were derived by caesarean section on day 114 of gestation. The newborn piglets were euthanatized right after birth. The gnotobiotic piglets were maintained in rigid tub isolators with flexible plastic canopy and were fed evaporated milk with vitamin D added ad libitum. Four piglets each were euthanatized on day 4 and 14. The remaining thirty piglets were divided into three groups of ten pigs each. The first group was maintained on milk diet while the second and third group were given solid food as a preweaning diet in addition to the evaporated milk starting on day 14 and were completely weaned to solid food on day 28. The solid food was a sterilized prescription diet (P/D) containing 8.5% minimum of crude protein, 5% minimum of crude fat, and 1% maximum of crude fiber normally used for dogs. On day 17, each piglet in the third group was given 30 ml per os of an inoculum consisting of 5 ml each of overnight broth cultures of selected microflora, i.e., Selenomonas ruminantium, Megasphaera elsdenii, Bacteroides sp., Lactobacillus acidophilus, Bifidobacterium adolescentis, and Acetivibrio ethanolignens. These organisms had been found in high numbers in the colon of conventional pigs 9 days post

\[\text{\textsuperscript{a}}\text{Carnation Company, Los Angeles, CA.}\]

\[\text{\textsuperscript{b}}\text{Hill's Division, Riviana Foods, KS.}\]
experimental inoculation with *Treponema hyodysenteriae* (I. Robinson, NADC, Ames, Iowa, unpublished data). Four, two, two, and two piglets from each group were euthanatized on day 28, 47, 50, and 54, respectively.

**Tissue Collection and Processing**

Before being sacrificed, the animals were anesthetized with intravenous pentobarbital sodium injection. Tissues from the proximal colon (2-3 cm distal to the ileocecal valve) and spiral colon (around the central flexure) were intraluminally fixed with cold 3% glutaraldehyde in cacodylate buffer. Tissue from the cecum was fixed by immersion in the same fixative. They were then trimmed, post-fixed in osmium tetroxide, dehydrated, and embedded in epoxy resin. Two blocks were randomly selected per each tissue. Sections one micrometer thick were cut, stained with toluidine blue, and examined by light microscopy. Size, shape, and staining intensity of goblet cells from the base of the crypt to the surface were evaluated. In order to characterize certain goblet cells ultrastructurally, tissues from selected blocks were trimmed, cut at 600-800 Å, and stained with 2% methanolic uranyl acetate and Reynold's lead citrate. Sections were examined by electron microscopy.
RESULTS

Newborn Unfed Piglets

There were no detectable morphological differences among goblet cells from the cecum, proximal colon, and spiral colon in toluidine blue stained sections.

Goblet cells at the base of the crypt generally stained indifferently compared to other epithelial cells. Occasionally, goblet cells with dark staining nuclei and cytoplasm were noted. At this level, goblet cells contained small numbers of mucin granules which were located in the apical part of the cells. Individual mucin granules were distinct and stained uniformly light purple. Their nuclei were round to slightly flattened and were basally located (figure 1). Ultrastructurally, the cytoplasm contained abundant free ribosomes, polyribosomes, and rough endoplasmic reticulum (RER). The RER was arranged in multiple interconnecting rows parallel to the lateral cell membranes and around the nuclear envelope. The cisternae of the RER was uniform in width and contained fine, dark, granular material. The mitochondria were scattered throughout the cytoplasm. Some mitochondria and RER were located in between the mucin granules (figure 2). The Golgi apparatus was prominent with numerous transfer vesicles and a few mucin granules in their vicinity. The mucin granules in the apical portion of the cell were uniform in size and were about one micrometer in diameter; most of them had the same level of electron density and their contents were finely granulated. Mucin
FIGURE 1. Proximal colon from newborn unfed piglet. Goblet cells at different levels of the mucosa. Most goblet cells stain indifferently from other epithelial cells. Pale goblet cells (arrow) are occasionally noted. Toluidine blue stain. 800 X.
granules in the vicinity of the Golgi apparatus, interpreted to be newly synthesized ones, were slightly smaller and lighter in electron density (figure 2). Microvilli were few in number and irregularly distributed. They were present on the apical surface only at the periphery of the cell opening into the crypt lumen. The lateral cell membranes generally did not interdigitate with neighboring cells and were connected with neighboring crypt epithelial cells by a few desmosomes (figure 3). The intercellular spaces were mildly dilated in two out of five newborn unfed piglets.

From the level slightly above the base of the crypt, goblet cells acquired their typical wine goblet appearance with a narrow base attached to the basement membrane and numerous mucin granules in the apical portion of the cells. The nuclei were basally located and generally round to slightly flattened. As the cells moved toward the surface, the nuclei were slightly oval. The mucin granules stained variably. Frequently, darker staining granules were located in the supranuclear region closer to the nuclei. The majority of goblet cells in this location stained indifferently from the neighboring epithelial cells (figure 1). Ultrastructurally, these goblet cells contained less abundant RER than those goblet cells located at the base of the crypt and most of the RER was dislocated toward the lateral cell membranes. The cytoplasm generally had the same level of electron density compared to other epithelial cells. However, occasional goblet cells with highly electron dense cytoplasm were noted. Intermediate filaments were not detected in goblet cells at
FIGURE 2. Cecum from newborn unfed piglet. Goblet cells at the base of the crypt with abundant RER (arrow), prominent Golgi apparatus (G), numerous transfer vesicles, and uniform mucin granules (M). Nascent mucin granules (arrow head) are smaller and of low electron density. Inset-transfer vesicles fusing with mucin granules. Bar = 2 μm.

FIGURE 3. Cecum from newborn unfed piglet. Goblet cell at the base of the crypt. It is connected to the neighboring epithelial cells by a few desmosomes (arrow). The lateral cell membranes do not interdigitate with neighboring cells. Bar = 1 μm.
this level. The lateral cell membranes slightly interdigitated with neighboring epithelial cells.

Small numbers of goblet cells with light staining cytoplasm were present in the mid crypt epithelium. These pale goblet cells appeared to be richer in cytoplasm than other goblet cells (figure 1). They contained a small amount of free ribosomes and RER. The RER was fragmented and slightly dilated. Connections between RER and the nuclear envelope were occasionally observed. The Golgi apparatus was prominent in these cells. Fine intermediate filaments were occasionally detected in the cytoplasm of these pale goblet cells (figure 4).

Toward the mouth of the crypt, goblet cells were elongated and contained oval and basally located nuclei. The longitudinal axis of the nuclei was generally perpendicular to the basement membrane. The nuclei and cytoplasm stained indifferently from the neighboring epithelial cells. The mucin granules stained heterogeneously with toluidine blue. Electron microscopically, these goblet cells contained less RER than goblet cells at the base of the crypt. Occasionally, a cluster of mucin granules extending from the Golgi apparatus to the base of the goblet was noted. These mucin granules were more electron dense than those mucin granules in goblet cells at the base of the crypt and were not granulated. The Golgi apparatus was well-developed with small numbers of nascent mucin granules and transfer vesicles in their vicinity (figure 5).

Surface goblet cells from all three sites of the large intestine
FIGURE 4. Proximal colon from newborn unfed piglet. Pale goblet cells at mid crypt with sparse, fragmented, and dilated RER (arrow), prominent Golgi apparatus (G), and heterogeneous mucin granules (M). Fine intermediate filaments (arrow head) are occasionally noted in the pale cytoplasm. Bar = 1 µm.

FIGURE 5. Proximal colon from newborn unfed piglet. Goblet cell at the crypt mouth with prominent Golgi apparatus (G) and heterogeneous mucin granules (M). The granules near the Golgi apparatus are of high electron density and are not granulated. Bar = 2 µm.
were smaller than those in the crypt. Some of them had a thin rim of cytoplasm around their goblet. This rim of cytoplasm was less electron dense than neighboring epithelial cells and rich in intermediate filaments arranged in random directions. Mucin granules were heterogeneous in both size and electron density. The nuclei were often indented. Mitochondria were more prominent near the basement membrane and around the goblet.

In one of the five newborn unfed piglets, several goblet cells in the mid crypt of the cecum, proximal colon, and spiral colon were extensively swollen. The RER was markedly dilated. Mucin granules lost their staining characteristics and the membranes of the granules were disintegrated.

Milk-fed Gnotobiotic Piglets

The morphology of goblet cells in the proximal and spiral colon was generally similar to those described in newborn piglets.

Numerous large swollen goblet cells were observed in the crypts of the cecum in 4 out of 8 milk-fed gnotobiotic piglets aged 4 to 14 days and 9 out of those aged 28 to 54 days. These swollen goblet cells were generally present from the level slightly above the base of the crypt to the level slightly below the crypt mouth (figure 6). These swollen goblet cells were noted only in moderate numbers in the proximal colon of one 54 day-old pig. No swollen goblet cells were detected in the spiral colon of any of the pigs. The frequency of occurrence of these swollen goblet cells is shown in table 1.
TABLE 1. The occurrence of swollen goblet cells in the crypt epithelium of the large intestine of newborn unfed and gnotobiotic piglets

<table>
<thead>
<tr>
<th>AGE (days)</th>
<th>Milk</th>
<th>P/D</th>
<th>P/D + Anaerobes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CE(^a)</td>
<td>PR(^b)</td>
<td>SP(^c)</td>
</tr>
<tr>
<td>0</td>
<td>1/5(^d)</td>
<td>1/5</td>
<td>1/5</td>
</tr>
<tr>
<td>4</td>
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<td>28</td>
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<tr>
<td>47</td>
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</tr>
<tr>
<td>50</td>
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<td>0/2</td>
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<tr>
<td>54</td>
<td>2/2</td>
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<td>0/2</td>
</tr>
<tr>
<td>28-54</td>
<td>9/10</td>
<td>1/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

\(^a\)CE = Cecum.

\(^b\)PR = Proximal colon.

\(^c\)SP = Spiral colon.

\(^d\)Number of pigs with swollen goblet cells over number examined.

\(^e\)ND = Not done.
FIGURE 6. Cecum from 54 day-old milk-fed gnotobiotic piglet. Swollen goblet cells (arrow) at the mid crypt from the level of slightly above the base of the crypt to the level slightly below the crypt mouth. Toluidine blue stain. 130 X.

FIGURE 7. Cecum from 14 day-old milk-fed gnotobiotic piglet. Swollen goblet cell with minimal recognizable fragmented and dilated RER (arrow) and mitochondria (M). The membranes of the mucin granules are disintegrated and mucin granules coalesce. The amorphous mucinous material bulges into the lumen. Bar = 2 μm.
Histologically, these swollen goblet cells had markedly pale and occasionally vacuolated cytoplasm. The individual mucin granules in most of these cells were not distinctly noticeable and the mucin contents appeared as a light purple staining amorphous material (figure 6). Occasionally, a stream of mucus extending from the goblet cells into the crypt lumen was noted. A few goblet cells with pyknotic nuclei and amorphous mucin content, interpreted to be necrotic goblet cells, were scattered among these swollen goblet cells. Electron microscopically, the swollen goblet cells had markedly swollen cytoplasm with minimal recognizable organelles. Among these were small numbers of slightly swollen mitochondria and fragmented and markedly dilated RER. The swollen mitochondria often accumulated in the infranuclear region. Membranes of the mucin granules were not clearly distinct. The mucin contents coalesced and appeared as an amorphous material of low electron density. This amorphous material often bulged into the crypt lumen. The nuclei were slightly swollen with mild margination of the nuclear chromatin. These swollen goblet cells maintained close contact with the neighboring epithelial cells and the basement membrane (figure 7).

Some swollen goblet cells contained only a few intact granules (figure 8a) while the others had considerable numbers of mucin granules loosely arranged in the markedly swollen cytoplasm (figure 8b).
FIGURE 8. Cecum from 14 day-old milk-fed gnotobiotic piglet.

a. Swollen goblet cell with a few intact mucin granules (M) and severely dilated RER (arrow). Bar = 2 μm.;
b. Swollen goblet cell with mucin granules (M) loosely arranged in the markedly swollen cytoplasm. Bar = 2 μm.

FIGURE 9. Proximal colon from 56 day-old, P/D-fed, gnotobiotic piglet with selected microflora. Surface goblet cells with a small number of intracellular mucin granules and a prominent cytoplasmic rim containing numerous intermediate filaments (IF). RER (arrow head) is sparse, fragmented, and slightly dilated. A few dense bodies (D) are located near the nucleus. Bar = 1 μm.
P/D-fed Gnotobiotic Piglets

The morphology of goblet cells in piglets on P/D was similar to those described in milk-fed ones. There were no detectable morphological differences between the goblet cells from piglets on P/D with or without selected microflora. As shown in table 1, it is evident that the swollen goblet cells occurred in the cecum of only 5 out of 10 piglets on P/D without selected microflora, while 9 out of 10 milk-fed piglets and 9 out of 10 P/D-fed piglets with selected microflora of the same age carried these swollen goblet cells in the crypt of the cecum. Only one, 50 day-old, P/D-fed piglet without selected microflora had these swollen goblet cells in the proximal colon. The numbers of swollen goblet cells in the colon were also much less than those found in the cecum.

Goblet cells with a prominent cytoplasmic rim around the goblet were present on the surface mucosa of all three sites of the large intestine from piglets on P/D either with or without selected microflora at a higher incidence than those in milk-fed piglets. They usually had lighter staining cytoplasm and a smaller amount of intracellular goblet cell mucin. Ultrastructurally, the cytoplasm was much lighter in electron density than the neighboring epithelial cells and contained numerous intermediate filaments. These intermediate filaments were arranged in random directions. RER was sparse, fragmented, and slightly dilated. A Golgi apparatus was not prominent. Mitochondria often clustered around the goblet which was made up of mucin granules of different size and electron density. The nuclei
were frequently indented. Lateral cell membranes were slightly interdigitated with those of neighboring cells. Frequently, there were a few dense bodies interpreted to be autophagosomes in these surface goblet cells (figure 9).
DISCUSSION

The morphology of goblet cells in newborn piglets was similar to that described in other animal species. However, in this study, goblet cells in gnotobiotic piglets had variable levels of electron density compared to other epithelial cells. This is in contrast to previous reports from other species in which goblet cells were more electron dense. The abundant quantity of RER and prominent Golgi apparatus present in most goblet cells were interpreted to indicate high metabolic activity of these cells because RER has been shown to have a central role in cellular biosynthesis of macromolecules including lipids, proteins, and complex carbohydrates. The Golgi apparatus of goblet cells has been shown to be a site of carbohydrate incorporation and sulfation. The shapes of goblet cells and their nuclei depend on the amount of mucin granules accumulated and the thickness of the epithelium. As goblet cells moved toward the surface where the epithelium was thicker, the goblet cells and their nuclei became more elongated. This is in agreement with previous observations.

Pale goblet cells have been previously noted in the small intestinal epithelium of rats and mice. No suggestion has been made with regard to the significance of these pale goblet cells. In newborn and gnotobiotic piglets, these pale goblet cells were occasionally present in the cecum, proximal colon, and spiral colon and were primarily scattered among epithelial cells in the mid crypt region. The low electron density of the goblet cell cytoplasm was
interpreted to be due to the lower quantity of RER and free ribosomes. It is generally known that mucin is composed of a protein core that is most likely produced in the RER. The small amount of RER was interpreted to indicate that these goblet cells are less active in mucin synthesis than other goblet cells. Since these cells are scattered among others, it may suggest that goblet cells are not equally active as they move from the bottom of the crypt to the surface and the pale ones may represent a less active form of mucin producing cells.

Neutra suggested that mucin goes through a process of concentration after synthesis. Whether this concentration occurs in the Golgi apparatus or after the mucin granules are released from the Golgi apparatus is not known. The more electron dense nature of the mucin granules in the vicinity of the Golgi apparatus in cells near the crypt mouth and surface suggests that mucin within the granules of these goblet cells is more concentrated than that produced by goblet cells at the base of the crypts. We believe that mucin concentration most likely occurs in the Golgi apparatus. As goblet cells migrate from the base of the crypt, the quantity of RER appeared to be reduced. Therefore, the more concentrated nature of mucin in the goblet cells at a higher level in the mucosa was thought to be a result of slower rate of mucin synthesis providing more time for the concentration to take place.

Goblet cell cytoplasm contains microtubules and intermediate filaments which are involved in maintenance of cell shape and
translocation of mucin granules from the Golgi apparatus into the
goblet. Many surface goblet cells in our gnotobiotic pigs had a
prominent cytoplasmic rim containing abundant intermediate filaments.
The presence of microtubules was not confirmed. We believe that all
goblet cells have intermediate filaments, but these are easier to
detect when the cytoplasm is of low electron density. The higher
incidence of these goblet cells with a prominent cytoplasmic rim on
the surface of the large intestinal epithelium from pigs on solid food
with or without selected microflora may be a result of cytoplasmic
contraction after most mucin granules have been released in response
to the introduction of solid food.

Many of the gnotobiotic piglets in this study had large swollen
goblet cells in the crypts of the cecum. We do not believe these
large swollen goblet cells to be an autolytic change because the
animals were anesthesized and tissues were collected and fixed
immediately. In addition, there is no evidence of any autolytic
changes in other epithelial cells. The occurrence of swollen goblet
cells only in the mid crypt region but not at the base of the crypt or
on the surface of the cecum also suggests that post mortem autolysis
is unlikely. In addition, these swollen goblet cells were present
only in the proximal colon of only 2 piglets and there were no swollen
goblet cells in the spiral colon. It is possible that these swollen
goblet cells are a special feature of the developing cecum in
gnotobiotic piglets.

In most of these swollen goblet cells, the mucin granule
membranes were disintegrated and the mucin contents coalesced and appeared as an amorphous material. Some of these cells had a stream of a secretory product extending from the goblet into the crypt. These swollen cells may, therefore, represent goblet cells under accelerated mucin release in response to unknown stimuli. Accelerated goblet cell mucin release has been induced by histamine and acetylcholine in intestinal mucosal explants. Only crypt goblet cells, but not surface goblet cells, responded to these stimuli. Failure of goblet cells to respond as they moved out of the crypt onto the surface is thought to be related to goblet cell maturation involving a loss or masking of plasma membrane receptors. The presence of responsive goblet cells only in the crypts is similar to the distribution of large swollen goblet cells as noted in this study. The relatively low electron density of the swollen goblet cells in this study, however, is in contrast with previous reports in which massively released goblet cells generally had darker cytoplasm than other epithelial cells. In addition, the absence of cavitation in goblet cells from our study did not support the idea of accelerated mucin secretion.

The presence of normal appearing goblet cells on the surface probably indicates that this swelling process is reversible since these goblet cells originate from the base of the crypt. We also believe that small numbers of these swollen goblet cells can undergo necrosis because some goblet cells with pyknotic nuclei were detected among the swollen ones.
The substance that caused goblet cells to swell is not determined. Since gnotobiotic animals are devoid of normal microflora, the microbial fermentation does not exist and many of the substances that would have been fermented by microflora may be present in the cecum. The presence of swollen goblet cells in the proximal colon of one piglet on milk diet and one piglet on solid food without selected microflora may be a result of spreading of an undetermined factor from the cecum.

We currently cannot explain the presence of swollen goblet cells at all three sites of the large intestine in only one out of five newborn unfed piglets. The more frequent occurrence of swollen goblet cells in this animal which had never received any food suggests that the mechanism responsible for the occurrence of swollen goblet cells may be different from those previously discussed.

Piglets on solid food without selected microflora had a lower incidence of swollen goblet cells than those on milk or solid food with selected microflora. It is known that certain dietary fibers can absorb materials, particularly water, certain minerals, and other organic materials, such as bile salts. In addition, some dietary fibers that increase stool weight are likely to decrease the intestinal transit time as well. The higher incidence of swollen goblet cells in P/D-fed piglets with selected microflora compared to those without selected microflora was interpreted to be related to the ability of certain anaerobes to digest dietary fibers.

In conclusion, the goblet cells from the large intestinal mucosa
of gnotobiotic piglets were similar to those previously described in other animal species except for their staining characteristics. Pale goblet cells occasionally occurred in the crypts of all three intestinal sites investigated and were thought to represent a less active form of goblet cells. Many gnotobiotic piglets used in our study carried numerous large swollen goblet cells in the mid crypt region of the cecum. The significance of these swollen goblet cells was not determined. The incidence of swollen goblet cells was lower in the group of animals on solid food without selected microflora than those on milk and solid food with microflora. Surface goblet cells with prominent cytoplasmic rim were more common in piglets on solid food with or without selected microflora than those on milk diet. The changes in goblet cell morphology in response to solid food and selected microflora were subtle. We do not believe that they impact on susceptibility of pigs to Treponema hyodysenteriae.
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SECTION III. THE PRESENCE OF VILLUS-LIKE STRUCTURES, VACUOLATED EPITHELIAL CELLS, AND FAT DROPLETS IN THE LARGE INTESTINAL MUCOSA OF NEWBORN AND GROWING GNOTOBIOTIC PIGLETS
THE PRESENCE OF VILLUS-LIKE STRUCTURES, VACUOLATED CELLS, AND FAT DROPLETS IN THE LARGE INTESTINAL MUCOSA OF NEWBORN AND GROWING GNOSTOBIOTIC PIGLETS


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SUMMARY

The cecum, proximal colon, and spiral colon from five newborn unfed and twelve milk-fed gnotobiotic piglets aged 4, 14, and 28 days old were investigated. Surface epithelial cells with apical tubular and vacuolar system (vacuolated cells) were present in the luminal two-thirds of villus-like structures present in all three large intestinal sites from the newborn-unfed piglets. The villus-like structures were still present on day 4 of life but were shorter than those in the newborn ones. There were no vacuolated cells present at 4 days of life. Fat droplets were present in high numbers in the surface columnar absorptive cells of the large intestine at all three sites on day 4 but were sporadic in the cecum and proximal colon of 2 out of 4 piglets on day 14. Occasional non-membrane bounded fat droplets were also found in the intercellular spaces indicating that absorbed fat was transported out of the epithelial cells.
INTRODUCTION

Neonatal piglets acquire passive immunity from their dams after birth by the absorption of colostral antibodies. This colostral antibody transfer generally occurs only in the first 24 to 48 hours after birth. Cessation of antibody transfer (closure) coincides with the loss of epithelial cells with cytoplasmic tubules and vacuoles from the duodenum and jejunum. In the ileum, epithelial cells with cytoplasmic tubules and vacuoles persist until the third week of life.

Wooding et al. (1978) reported the presence of vacuolated epithelial cells on the villus-like structures of the colonic mucosa of newborn piglets. They also have shown that these vacuolated colonic epithelial cells can readily take up colostrum injected directly into the large intestine. They, however, thought that these cells must have been lost before any significant amount of colostrum reached the colon, and questioned the role of these vacuolated cells in the colostral antibody absorption.

Apart from the occurrence of villus-like structures and vacuolated epithelial cells, fat droplets have been observed in the large intestinal mucosa of newborn conventional and gnotobiotic piglets. This finding indicates that the porcine large intestine may participate in fat absorption in the early days of life.

The objectives of this study were: i) to evaluate the presence of vacuolated cells and the villus-like structures in the large intestinal mucosa of newborn unfed piglets, and ii) to evaluate the
presence of fat droplets in the columnar absorptive cells of the large intestine in milk-fed gnotobiotic piglets within the first four weeks of life.
MATERIALS AND METHODS

Five newborn unfed and twelve milk-fed gnotobiotic piglets were used in this study. The detailed experimental design has been previously described. Briefly, newborn unfed piglets were euthanatized immediately after birth. Four milk-fed gnotobiotic piglets each were euthanatized on day 4, 14, and 28. Tissues from the cecum, proximal colon, and spiral colon were collected. For light microscopy, tissues were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin blocks. Sections five micrometers thick were cut and stained with hematoxylin and eosin. Additional tissues were frozen, embedded, sectioned with a cryostat at 6 micrometers thick, and stained with oil red 0 to demonstrate absorbed fat in the columnar epithelial cells.

Adjacent tissues were fixed in cold 3% glutaraldehyde in cacodylate buffer, post-fixed in osmium tetroxide, dehydrated, and embedded in epoxy resin. Sections one micrometer thick were cut and stained with toluidine blue to demonstrate vacuolated epithelial cells and intracellular fat droplets. Sections were then examined by light microscopy. Selected blocks were trimmed and cut with an ultramicrotome at 600–800 A°. Sections were stained with 2% methanolic uranyl acetate and Reynold's lead citrate and examined by electron microscopy.
RESULTS

Newborn Unfed Piglets

In the cecum, proximal colon, and spiral colon of newborn unfed piglets, there were numerous villus-like structures present on the mucosal surface (figure 1). The length of these villus-like structures varied and depended greatly on the degree of the large intestinal contraction. In contracted pieces of tissues, the villus-like structures were easily recognized and were as long as 280 micrometers in length. In uncontracted pieces, however, these villus-like structures appeared as small protuberances. With toluidine blue stain, the luminal two-third of the continuous epithelial cells between adjacent crypts were consistently vacuolated (figure 1). Multiple vacuoles were noted in each epithelial cell and many of these vacuoles contained dark-blue staining material. These vacuolated epithelial cells occurred in all three sites of the large intestine studied, but they were not easily detected by HE stain.

Electron microscopically, the vacuolated epithelial cells in the cecum, proximal colon, and spiral colon were identical and characterized by a prominent apical tubular and vacuolar system. Some of these tubular structures were connected with plasma membrane invaginations at the base of the microvilli. Beneath these tubular structures, there were considerable numbers of variable-sized supranuclear vacuoles (figure 2). Occasional infranuclear vacuoles were also noted (figure 3). These vacuoles were membrane bounded and
FIGURE 1. Spiral colon from newborn unfed piglet. Numerous villus-like structures with vacuolated epithelial cells (arrow) on the luminal two-thirds. Toluidine blue stain. 180 X.

FIGURE 2. Spiral colon from newborn unfed piglet. Epithelial cell with a prominent apical tubular and vacuolar system. Some tubules are connected to plasma membrane invaginations at the base of the microvilli (arrow). The microvilli are shorter than those in the neighboring non-vacuolated epithelial cells. Bar = 1 μm.
most of them contained loose granular material. Most of the large vacuoles also contained irregular clumps of highly electron dense material which was usually located around the periphery of the vacuoles in contact with the limiting membranes. The typically vacuolated cells usually had shorter microvilli compared to the neighboring non-vacuolated epithelial cells (figure 2). The mitochondria and endoplasmic reticulum were distributed throughout the cytoplasm. In some vacuolated epithelial cells, however, the mitochondria were more densely packed underneath the terminal web (figure 3). The Golgi apparatus was not commonly noted.

Apart from these vacuolated cells, there were some epithelial cells with a prominent apical tubular system which contained no vacuoles (figure 4). Occasional epithelial cells with well developed apical tubular system and small supranuclear vacuoles filled with exclusively electron-dense material were noted (figure 5).

There were no fat droplets detected by either oil red O stain or electron microscopy in any of the columnar absorptive cells of either the cecum or the colon of newborn unfed piglets.

Four-day-old Gnotobiotic Piglets

At four days of life, the villus-like structures were still present in the cecum, proximal colon, and spiral colon but they were much shorter than those in newborn unfed piglets (figure 6). A few of them were as long as 200 micrometers, but most were less than 100 micrometers. The vacuolated cells were not detectable either by
FIGURE 3. Proximal colon from newborn unfed piglet. Epithelial cells with a prominent apical tubular and vacuolar system. Some vacuoles are located in the infranuclear region (*). Bar = 2 μm.

FIGURE 4. Cecum from newborn unfed piglet. Epithelial cell with a prominent apical tubular system which contains no vacuoles. Bar = 1 μm.
FIGURE 5. Cecum from newborn unfed piglet. Epithelial cells with a prominent apical tubular system and small vacuoles containing exclusively high electron dense material (arrow). The intercellular spaces (S) are widely opened. Bar = 1 μm.

FIGURE 6. Cecum from 4 day-old milk-fed gnotobiotic piglet. Villus-like structures present in the large intestinal mucosa. Hematoxylin and eosin stain. 190 X.
toluidine blue stain or electron microscopy. Most of the surface epithelial cells were loaded with numerous variable-sized droplets. These droplets were easily detected by either toluidine blue (figure 7) or oil red 0 stain (figure 8). With toluidine blue stain, they were greenish-yellow in contrast to the light blue staining of the cytoplasm. With oil red 0, they stained distinctly red, and were interpreted to be fat droplets. Most of these fat droplets were located in the supranuclear region between the nucleus and the apical cell surface. Infranuclear fat droplets were also noted. There were no detectable differences between the cecum, proximal colon, and spiral colon.

Electron microscopically, the cytoplasm was filled with multiple droplets of low electron density. The majority of them were located in the area between the terminal web and the nucleus. They were non-membrane bounded. Their size was highly variable and ranged from 0.1 to 6 micrometers. Most of the small droplets clustered in the perinuclear region (figure 9). Occasionally, clusters of non-membrane bounded fat droplets ranging in size from 60 to 180 nm were found in the intercellular spaces (figure 10).

Fourteen-day-old Gnotobiotic Piglets

At this age, very few intracellular droplets were detected by either oil red 0 or toluidine blue stain. Occasional oil red 0 positive (fat) droplets were noted but only in the cecum of one piglet while toluidine blue stain demonstrated greenish-yellow droplets in
FIGURE 7. Proximal colon from 4 day-old milk-fed gnotobiotic piglet. Surface epithelial cells containing numerous, varying sized droplets (arrow) interpreted to be absorbed fat. The majority of the droplets are in the supranuclear region. Toluidine blue stain. 450 X.

FIGURE 8. Spiral colon from 4 day-old milk-fed gnotobiotic piglet. Oil red O-positive material (arrow) in the surface epithelial cells at the tip of the villus-like structures. Oil red O stain. 190 X.
FIGURE 9. Cecum from 4 day-old milk-fed gnotobiotic piglet. Surface epithelial cells containing numerous non-membrane bounded droplets of varying size and of low electron density (fat) (*). Most droplets are located in the supranuclear location. Bar = 2 µm.

the cecum and proximal colon of two piglets. These droplets were present sporadically only in the surface epithelial cells. Within each cell, there were fewer droplets than those in the surface epithelial cells of the four-day-old piglets (figure 11). Ultrastructurally, these droplets of low electron density were identical to those in the four-day-old piglets. However, the number of droplets per cells was small and the size of single droplets ranged from 0.3 to 3 micrometers (figure 12).

Twenty-eight-day-old Gnotobiotic Piglets

There were no detectable fat droplets present in any of the columnar absorptive cells of the cecum, proximal colon, and spiral colon of any piglets at this age. The morphology of the columnar absorptive cells was identical to those previously described.\textsuperscript{5}
FIGURE 11. Cecum from 14 day-old milk-fed gnotobiotic piglet.
Very few fat droplets in occasional surface epithelial cells (arrow). Toluidine blue stain. 450 X.

FIGURE 12. Proximal colon from 14 day-old milk-fed gnotobiotic piglet. Small numbers of non-membrane bounded fat droplets in the cytoplasm of surface epithelial cells (*). Bar = 2 μm.
DISCUSSION

The morphology of the large intestinal epithelial cells with an apical tubular and vacuolar system as noted in our study is generally identical to those described in the ileum and colon of neonatal piglets. However, we also noted some vacuolated epithelial cells with a prominent apical tubular system which contained no vacuoles, and certain cells with a well developed apical tubular system and relatively small vacuoles filled with dark electron-dense material. The significance of these uncommon cell types was not determined. We, however, believe that these cells may represent different stages of cellular differentiation toward vacuolated cells with a prominent tubular and vacuolar system.

In contrast to a previous observation, the vacuolated cells noted in this study were more uniformly distributed in the colon. We found these cells to be evenly distributed in sections of proximal and spiral colon, and to commonly occupy the luminal two-thirds of the villus-like structures. In addition, these cells were also present in the cecum of newborn piglets. Although the villus-like structures were still present on day 4 of life, the vacuolated cells were not detected at this age. The exact time of disappearance and the significance of the vacuolated epithelial cells and the villus-like structures were not determined. According to Staley et al. (1970), villus-like structures are still present in the colon of six-day-old gnotobiotic piglets. In reviewing their photomicrograph, the length of the villus-like structures appears to be much shorter than those in
our newborn piglets but corresponds well with our observation on day 4. Wooding et al. (1978) reported that the vacuolated cells in the colon of pigs can readily take up colostrum injected directly into the large intestine. However, they thought that these cells are lost before protein reaches the colon in any quantity and proposed that these cells apparently have no direct function in postnatal life. We feel that the presence of vacuolated cells and villus-like structures in the cecum and colon are probably more than just "the result of a fortuitous and unpredictable longitudinal spreading of the factors which cause the ileal type of differentiation." Since these vacuolated cells can take up colostrum, it seems reasonable to assume that the presence of villus-like structures may facilitate colostral absorption by providing more surface area for absorption during the first hours of life. The functional role of these vacuolated cells in colostrum absorption needs further evaluation.

The presence of fat droplets in colonic surface epithelial cells has been observed in six-day-old gnotobiotic piglets and in conventional piglets no longer than two days after birth. In this study, we noticed oil red 0 positive droplets which were interpreted to be absorbed fat in the columnar absorptive cells of both the cecum and colon regularly up to day 4 and occasionally up until the animals were fourteen days old. However, at fourteen days of age, only the cecum and proximal colon of 2 out of 4 piglets were shown to contain fat, and the occurrence of fat droplets was very sporadic. Normally, dietary fats are completely digested and absorbed in the small
intestine, but if absorption is incomplete, fats enter the colon.\textsuperscript{8} Surface epithelial cells of the colon may absorb fatty acids, resynthesize triglycerides, form chylomicrons, and release them into the lamina propria, although the process is not as efficient as in the small intestine.\textsuperscript{8} Monoglycerides and free fatty acids are chiefly absorbed in the duodenum and the upper jejunum.\textsuperscript{9}

Why oil red 0 did not detect fat droplets in the cecum of one and proximal colon of two 14 day-old piglets that were shown to be positive by toluidine blue and electron microscopy is not known. We believe, however, that since the oil red 0 stain was applied on frozen sections and very few droplets were present in the large intestinal epithelial cells at this age, they may have been washed off in the process of staining.

The fat droplets present in the large intestinal epithelium are non-membrane bounded and are similar to those previously described in the colon of conventional piglets\textsuperscript{5} and in the terminal ileum of rats.\textsuperscript{10} These non-membrane bounded fat droplets were also detected in the epithelial cells of the small intestine when high doses of fat were administered to mice. Absorbed free fatty acids and monoglycerides are normally resynthesized to triglycerides in the columnar absorptive cells.\textsuperscript{9,10} The amount of absorbed monoglycerides and free fatty acids that exceeded the rate of triglyceride synthesis resulted in a non-physiological flooding of the cells.\textsuperscript{11,12} In rats, the non-membrane bounded fat droplets in the cytoplasm of the columnar cells of the terminal ileum were thought to
be related to the known reduced rate of triglyceride synthesis in this region. It is not known whether the presence of non-membrane bounded fat droplets in the cytoplasm of large intestinal epithelial cells reflects the low rate of triglyceride synthesis in this region or indicates the higher rate of free fatty acids and monoglyceride absorption.

Although fat droplets have not been detected in colonic mucosa of newborn conventional piglets after they were two days old, the colon can still take up fat at 10 days of life if fat is artificially injected directly into the large intestine. Therefore, the failure to detect fat droplets in the colonic epithelial cells of conventional piglets was interpreted to be a result of rapid increase in fat absorption by the small intestine. In our study, fat droplets in the columnar absorptive cells, believed to be absorbed fat, were demonstrated in the cecum and colon of gnotobiotic piglets until day 14, much longer than in the conventional ones. The prolonged presence of fat droplets in the large intestinal epithelial cells of gnotobiotic piglets may reflect a slower increase in triglyceride synthesis efficiency in the large intestinal enteroabsorptive cells of gnotobiotic animals resulting in flooding of the cytoplasm with monoglycerides and fatty acids. Alternately, it may reflect a slower increase in fat absorption efficiency with age in the small intestine resulting in a higher amount of fat available for absorption in the large intestine. Even though it has been shown in rats that oleic acid absorption was the same in gnotobiotic animals as in conventional
ones,\textsuperscript{13,14} the surface area of the small intestinal mucosa of the former is much less than that of the latter ones.\textsuperscript{15} This difference in the surface area of the small intestine may result in a more efficient fat absorption. The total surface area of the small intestine of gnotobiotic piglets in comparison to conventional ones is not known. Lipase and conjugated bile salts play an important role in fat digestion and absorption in the intestine.\textsuperscript{9,10} In the large intestine of conventional animals, 95\% of bile acids are non-conjugated while 95\% of bile acids in gnotobiotic animals are conjugated.\textsuperscript{16} Lipase activity in cecal and colonic contents of gnotobiotic animals is also higher than that in conventional ones.\textsuperscript{17}

The detection of fat droplets in the intercellular spaces is in agreement with a previous report\textsuperscript{5} and probably indicates that fat absorbed by the cecal and colonic epithelial cells can be transported out of the cells into the intercellular spaces.

In conclusion, the cecum and colon of newborn piglets contained vacuolated epithelial cells on the villus-like structures. The significance of these two structures awaits further investigation. Large intestinal epithelial cells of the newborn gnotobiotic piglets contained fat droplets up until day 14 of life. Some of these droplets were transported into the intercellular spaces. The extent of fat absorption and transportation by the large intestinal epithelial cells of pigs needs further evaluation. This fat absorption and transport ability may be important to the animals when normal small intestinal fat absorption and processing are impaired.
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SUMMARY AND DISCUSSION

In the cecum, proximal colon, and spiral colon of newborn unfed piglets, there were numerous villus-like structures present on the mucosa. The luminal two-thirds of these villus-like structures were covered by epithelial cells with a prominent apical tubular and vacuolar system (vacuolated epithelial cells). These vacuolated epithelial cells have been shown to take up colostrum injected directly into the large intestine.\textsuperscript{14} The presence of the vacuolated epithelial cells and villus-like structures in newborn piglets may be important for absorption and transfer of colostral antibodies during the first hours of life and needs further evaluation.

Goblet cells from the cecum, proximal colon, and spiral colon were identical and were similar to goblet cells from other species. However, in the large intestine of newborn piglets, goblet cells generally had the same level of electron density as other epithelial cells which is in contrast to the high electron density of goblet cells reported from other species.\textsuperscript{35,52,55,57} Occasionally, pale goblet cells, similar to those described previously in mice\textsuperscript{35} and rats,\textsuperscript{57} were found in the crypt epithelium. These pale goblet cells contained few RER and it was fragmented and slightly dilated. These cells were interpreted to be an inactive form of goblet cells. Some surface goblet cells had a prominent, low electron dense rim of cytoplasm around the mucin goblet. This cytoplasm was rich in intermediate filaments arranged in random directions. The intermediate filaments play a role in maintaining the shape of goblet cell as well as in
translocation of mucin granules from the Golgi region into the goblet.\textsuperscript{58,59} It was assumed that all goblet cells contained these intermediate filaments but they were more easily detected when the cytoplasm was of low electron density.

Histochemically, in newborn unfed piglets, only a small number of goblet cells at the base of the crypt produced sialomucin while goblet cells in the upper part of the crypt and on the surface produced sulfomucin. Sialomucin-producing goblet cells were more prominent in the cecum than in the proximal and spiral colon. This was similar to what has been previously described in rats, rhesus monkeys, and man, but was different from guinea pigs, hamsters, cats, and dogs.\textsuperscript{13}

The surface epithelium of the large intestine of gnotobiotic pigs, 4 and 14 days of age, contained droplets which were interpreted to be absorbed fat. These droplets were numerous in the surface epithelial cells of the cecum, proximal colon, and spiral colon of 4 day-old piglets but were sporadic in the cecum and proximal colon of 14 day-old piglets. They were not found in older piglets. Although fat droplets were not detected in the surface epithelial cells of the large intestine of conventional pigs after 2 days of life, the large intestinal epithelium of these animals can take up fat artificially injected into the large intestine at 10 days of age.\textsuperscript{14} The decrease in the number and finally absence of fat droplets from the large intestinal epithelial cells of gnotobiotic piglets in this study may reflect the marked increase in rate of triglyceride synthesis and chylomicron transportation in this part of the intestine or indicate an
increase in fat absorption efficiency in the small intestine resulting in a smaller amount of fat available for absorption in the large intestine.

The morphology of the goblet cells in the proximal and spiral colon of gnotobiotic piglets was similar to that in the newborn unfed piglets. In the cecum, however, large numbers of swollen goblet cells were found in the mid crypt epithelium of 4 out of 8 milk-fed piglets aged 4 and 14 days and 9 out of 10 of those aged 28 to 54 days. These swollen goblet cells are believed to be unique for the cecum of growing gnotobiotic piglets. They are not believed to be an autolytic change because the animals were anesthesized and tissues were collected and fixed immediately. In addition, there was no evidence of any autolytic changes in other epithelial cells. The distribution of these swollen goblet cells only in the crypt epithelium and not on the surface is similar to the distribution of goblet cells under accelerated mucin release. The relatively low electron density and the absence of cavitation in the apical part of these cells, however, did not support the idea of accelerated mucin secretion.

Even though there is no distinct morphological goblet cell difference in developing gnotobiotic piglets from 0 to 54 days, the degree of mucin sulfation decreased with age especially for the lower and upper parts of the crypts of the cecum and proximal colon. These decreases in degree of sulfation are believed to reflect a more rapid turnover rate of the goblet cells resulting in more immature goblet cells in the lower and upper parts of the crypts.
The introduction of solid food and selected microflora had minimal effect on the goblet cell morphology and no statistically significant effect on the degree of sulfation. Swollen goblet cells occurred in only 5 out of 10 piglets on solid food without selected microflora, while 9 out of 10 milk-fed piglets and 9 out of 10 piglets on solid food with selected microflora had these cells in the mid crypt epithelium of the cecum. The lower incidence of swollen goblet cells in piglets on solid food without selected microflora may be related to the capability of dietary fibers to absorb organic materials. Some dietary fibers that increase stool weight are also likely to decrease the intestinal transit time. The higher incidence of swollen goblet cells in piglets on solid food with selected microflora in comparison to those without selected microflora may be related to the ability of certain microflora to digest dietary fibers. The higher frequency of surface epithelial goblet cells with a prominent cytoplasmic rim and a small amount of intracellular mucin content in pigs on solid food either with or without selected microflora was interpreted to be an adaptation of the mucosa to the introduction of solid food.

Morphometrically, the introduction of solid food and selected microflora caused a marked drop in the amount of intracellular mucin content per unit volume of the large intestinal epithelium ($V_y$), and the effects were additive. After this temporary reduction, the $V_y$ increased gradually until day 54 when the experiment was terminated. The temporary drop in $V_y$ is most likely an adaptation to the introduction of solid food and selected microflora, while the gradual
increase in $V_v$ may reflect an increase of intracellular mucin accumulation or may reflect an increase in cell differentiation toward goblet cells.

The findings from this study with regard to the effects of age, diet, and selected microflora on the large intestinal mucosa could not explain the impact of these factors on the susceptibility to *Treponema hyodysenteriae*. The result must be interpreted with some precautions because of following reasons. First, gnotobiotic piglets have been used instead of the conventional ones. As generally known that the presence of microflora in the intestinal tract is important for the maintenance of "normal" intestinal morphology and physiology. Even though selected microflora has been inoculated in some of the animals to imitate the conventional condition, the numbers of strains of microflora used are limited compared to over 400 species of microorganisms that are normally present in the large intestinal lumen. Second, some of the gnotobiotic piglets were maintained on milk diet throughout the experiment. Some piglets have been fed solid food but this was a sterilized prescription diet normally used for dogs. The amount of dietary fiber present in the prescription diet is less than 1% which is much lower than the amount of dietary fiber present in normal pig feed. More research needs to be done in this area before the pathogenesis of swine dysentery and the susceptibility to *Treponema hyodysenteriae* are completely understood.
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