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Intestinal Cell Kinetics

Modulation caused by age, gender and microbial status in rats and mice. An experimental study in germfree, conventional, *Lactobacillus rhamnosus GG* and *Clostridium difficile* monoassociated animals

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To

……

With Best Regards

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To my beloved family,
and to my parents

SUMMARY

Mammals living in symbiosis with microbes create conventional organisms. The interactions or cross-talks that continuously occur demonstrate how microbes may influence on series of parameters, including intestinal cell kinetics and mucosal morphology. The aim of this study was to investigate modulation of intestinal cell kinetics and morphological parameters caused by age, gender, and microbial status in rats and mice. These studies were performed in germfree (GF), conventional (Conv), and ex-GF animals, mono-associated with either *Lactobacillus rhamnosus GG* (LGG) or *Clostridium difficile*. The first two studies were performed in GF and Conv rats and mice, aiming to evaluate the influence of gender, age and microbial status on epithelial cell kinetic and morphological parameters in different compartments of the intestinal tract. These investigations were performed in order to establish base-lines for future cell kinetic and morphological investigations. The mitotic index, growth fraction, number of cells in the crypts and villi, depth of the crypts, height of the villi and crypt/villus ratio were determined. In main, the results were as followed;

Rats: Gender: The effects were mainly expressed in the upper part of the small intestine (higher mitotic activity, more cells in the crypts and villi, and deeper crypts in males than in females).
Age: The effects were mainly upon morphological parameters in the distal part of the small intestine (deeper crypts, longer villi and more cells in the villi in the older rats).
Microbial status: The effects were somewhat scattered throughout the intestine. The most pronounced differences were longer villi and more cells in the villi in GF rats.

Mice: In main, influences of gender and age were as found in rats. However, the influence of microbial status was more pronounced in mice, especially in the large intestine.

Taken together, the results demonstrated that gender, age and microbial status influenced upon cell kinetics as well as intestinal mucosa morphology in rats and mice in a compartmentalised manner.

The following two studies composed association of young GF male rats with either a probiotic strain or a potentially pathogenic strain, aiming to study the influence of these microbes on cell kinetic parameters. LGG is used for production of fermented food products i.e., probiotics, aiming to promote health. *Cl difficile* is suggested to be one major cause of antibiotic-associated diarrhea.

The results from LGG inoculated rats showed that a short-term mono-association induced a compartmentalised increase in cell production and number of villus cells. Intestinal cell production is looked upon as a major regulatory and protective defence mechanism. It was assumed that this stimulatory effect could be of importance for explaining the protective and curative effects of LGG in rota-virus infections.

The results from rats mono-associated with a toxin producing strain of *Cl difficile* showed a marked influence on cell kinetics. After 3 days mono-association, a significant increase in cell production was observed in all compartments, followed by a dramatic reduction on day 7 and a shift to Conv values on day 21. Histological investigations revealed weak signs of damages. The rats looked healthy throughout the study period and no signs of diarrhea were observed. Increasing amounts of toxin were found throughout the intestinal tract during the study.

In another series of experiments, GF rats were mono-associated with a non-toxin producing strain of *Cl difficile*. A slight increase in intestinal cell production was seen after 3 days, but after 7 days no dramatic reduction was observed.

Our putative theory is that toxin producing *Cl difficile*, by temporally switching off the intestinal cell production, creates suitable attack points for unknown pathogens in the intestinal flora to cause symptoms and signs related to the so-called antibiotic-associated diarrhea.

Thus, intestinal microbes - physiological as well as pathophysiological strains - act on intestinal cell kinetics and morphology in a compartmentalised manner. Increased knowledge on such prokaryotic/eucariotic cross-talks creates possibilities for new prophylactic and therapeutic approaches. Gnotobiotic animals are irreplaceable models in these studies.

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ABBREVIATIONS

AGUS	A rat strain, of uncertain origin, inbred and reared at the Department of Germfree Research (later Laboratory of Medical Microbial Ecology) since 1956.
Conv	Conventional: A macroorganism with an undefined microflora.
Ecosystem	The relationship between living organisms in a defined area.
GAC	Germfree Animal Characteristic: The recording of any anatomical structure, biochemical, immunological or physiological function in a macroorganism, which has not been affected by the microflora.
GF	Germfree: A macroorganism free from all forms of outer life, i.e., bacteria, viruses, fungi and protozoa.
GI	Gastrointestinal
GrF	Growth Fraction: The portion of a cell population actively engaged in proliferation.
LGG	Lactobacillus rhamnosus GG
MAC	Microflora Associated Characteristic: The recording of any anatomical structure, biochemical, immunological or physiological function in a macroorganism, which has been affected by the microflora.
MI	Mitotic index: Calculated from the formula: MI = Nm/Nt x 100, where Nm is the number of mitotic cells and Nt is the total number of cells in the left column of the crypts.
NMRI/KI	A mouse strain transferred from the National Institutet of Health, Bethesda, USA, 1960, to the Department of Germfree Research, (later Laboratory of Medical Microbial Ecology), Karolinska Institutet.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their Roman numerals:

I **M. Banasaz**, M. Alam, E. Norin and T. Midtvedt. Gender, age and microbial status influence upon intestinal cell kinetics in a compartmentalised manner. An experimental study in germfree and conventional rats. Microbial Ecology in Health and Disease 2000; 12 (4): 208-218.

II **M. Banasaz**, E. Norin and T. Midtvedt. The role of gender, age and microbial status on cell kinetics in the gastrointestinal tract of mice. An experimental study in germfree and conventional mice. Microbial Ecology in Health and Disease 2001: 13 135-142.

III **M. Banasaz**, E. Norin, R. Holma, and T. Midtvedt. Increased enterocyte production in gnotobiotic rats mono-associated with *Lactobacillus rhamnosus* GG. Applied and Environmental Microbiology. In press, June 2002: 68(6).

IV **M. Banasaz**, L, Burman, E. Norin, T. Åkerlund, and T. Midtvedt. Mitotic activity in germfree rats mono-associated with toxin positive/negative *Clostridium difficile*. Manuscript.

INTRODUCTION

Intestinal microbial ecosystems

The intestinal ecosystem is by far the most complicated "system" within the mammalian body. It is shaped by a continuum of interactions between its microbes (both intra - and inter - species communications), and the host epithelium (cell turn over, mucosal immune system, microvasculature, and enteric nervous system) (*Gordon et al, 1997; Falk et al, 1998; Norin and Midtvedt., 2000*). The interactions between intestinal bacteria and their host can be viewed in terms of a functional entity that spans symbiosis, commensalism and pathogenicity (*Bry et al, 1996; Hooper et al, 1999 & 2000; Hooper and Gordon., 2001*). A symbiotic relationship is mutually beneficial for both partners, and a pathogenic relationship benefits one part at the expense of the other. The term commensalism literally means "at table together" and it is used to define relationships beneficial for one part without necessarily being detrimental to the other.

The intestinal microflora

Principally the intestinal microflora can be investigated in three different routes:

A. Which microbes are there?
B. What can the microbes do?
C. What have the microbes done?

Att. A**:**

Utilising classic and modern molecular methods, the complexity of the intestinal flora have been clearly demonstrated. It is well documented that the composition differs among species, within the same species and also during life within the same individual (*Savage 1977; Tannock 1995; Zoetendal at al, 2001*). The population level in various compartments is thought to be regulated by a competition for nutrients and space. It should be emphasised that a qualitative and quantitative evaluation of the microflora present in various intestinal compartments is expensive, extremely time-consuming and difficult to perform. The data in table 1 (*Rowland 1988)* show the composition of the intestinal microflora in mice and rats without regard to age and diet.

Table 1: Distribution and composition of the intestinal microflora in rats (R) and mice (M), (Log $_{10}$ bacteria per gram wet weight intestinal content).

Microflora	Proximal small intestine	Distal small intestine	Large intestine
Bacteroides	5-6 (R) 5-8 (M)	6-8 (R) 6-7 (M)	7-9 (R) 9-10 (M) 8.2 (R) 9.4 (M)
Bifidobacteria	5-7 (R) 6-8 (M)	6-8 (R) 7-8 (M)	8-9 (R) 8-9 (M)
Lactobacilli	6-7 (R) 7-8 (M) 7.0 (R) 6.3 (M)	6-7 (R) 7-8 (M) 8.0 (R) 8.0 (M)	8-9 (R) 8-9 (M) 8.4 (R) 8.8 (M)
Clostridia	2-3 (M)	2-3 (M)	0-6*(R) 0-3* (M) 2.0 (R)
Enterobac-teriaceae	3-5 (R) 3-6 (M) 1.7 (M)	3-5 (R) 3-6 (M) 4.5 (R) 4.0 (M)	5-7 (R) 4-6 (M) 5.2 (R) 5.4 (M)
Streptococci	4-5 (R) 3-4 (M) 3.0 (R) 5.0 (M)	2-6 (R) 4-7 (M) 5.0 (R) 6.7 (M)	5-7 (R) 6.8 (M) 6.0 (R) 6.0 (M)

*Not present in all individuals. Upper lines show data from Drasar et al (1970) and lower lines show data from Smith (1965).

Att. B:

Over the years, several methods have been established to study the functional capacity of the intestinal flora, *in vitro* as well as *in vivo*. Some of these methods, as the bile acid deconjugation test, the urease test and the lactulose test are well established in clinical practice. However, in main, the same difficulties as in A hold true also for this way of investigation.

Att. C:

In order to study what the flora has done, it is necessary to evaluate which mechanisms and reactions are related to the host and which are related to the flora itself, respectively. With a slight modification of terms first used by Claude Bernard, the host side of the ecosystem can be defined as *milieu interieur*. The non-host side as *milieu exterieur*, and both together as *milieu total* (*Midtvedt 1999*).

Studies in germfree (GF) individuals belonging to different species have contributed to established basal values for anatomical structures and physiological, biochemical, and immunological variables in the *milieu interieur*. When such baselines are established, the functions of the flora, as well as alterations in these functions, can be worked out. In these studies, the GAC/MAC concept (Germfree Animal Characteristic/ Microflora-Associated Characteristic) - has been shown to be of considerable value. A GF animal, i.e. the *milieu interieur* represents a summary of all GACs, and a similar collection of MACs describes a milieu total or a Conv animal. A simple equation - *milieu total* minus *milieu interieur* gives

milieu exterieur - or what the microbes have done. Over the years, several pairs of GAC/MAC has been described (*Midtvedt 1986; Falk et al, 1998; Norin and Midtvedt., 2000*). To simplify, a GAC/MAC switch represents a prokaryotic / eukaryotic cross-talk and a MAC is the phenotypic expression of this cross-talk. However, it should be emphasised that a cross-talk between a host and a single commensal bacterial strain (i.e., *Bacteroides thetaiotaomicron*) can modulate expressions of genes involved in several important intestinal functions, including nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation (*Hooper et al, 1999; 2000; Hooper and Gordon., 2001*).

Selected members of the intestinal microflora

Lactobacillus rhamnosus

The lactic acid bacteria comprise a taxonomically diverse group of gram-positive, non-sporeforming cocci and rods, usually catalase-negative, which grow under micro-aerophilic to strictly anaerobic conditions. These microbes are found to be present in the gastrointestinal (GI) tract of all mammals, and a number of strains in the group are also utilised as so-called probiotics. Among the probiotics, *Lactobacillus rhamnosus* GG (once known as *L.casei* GG, LGG) is the strain most thoroughly studied (*Salminen et al, 1993*). LGG was isolated from a healthy person by screening several stool specimens for isolates that were unaffected by acid and bile (*Alander et al, 1997*). The ability of LGG to survive passage through the GI tract has been demonstrated in both children and adults (*Goldin et al, 1992; Millar et al, 1993; Saxelin et al, 1993*). The claimed beneficial effects (*Siitonen et al, 1990; Isolauri et al, 1991; Salminen et al, 1996; Majamaa and Isolauri., 1997; Saxelin 1997*) such as prevention of antibiotic-associated diarrhea including treatment of relapsing *Clostridium difficile* (*CL. difficile*) diarrhea, prevention of acute diarrhea, influence upon the duration of rota-virus diarrhea in children (*Guarino et al, 1991; Guarino et al, 1994; Majamaa et al, 1995*), and enhancement of intestinal immunity (*Malin et al, 1996; Salminen et al, 1996; Saxelin et al, 1993*) as well as influence upon the atopic disease in children (*Kalliomäki et al, 2001*). However, the mechanisms behind these effects are still far from elucidated.

Clostridium difficile

Clostridium difficile is a sporeforming, gram-positive, catalase-negative, anaerobic rod. In 1935, it was described to be a normal and harmless commensal in feces from infants (*Hall and O-Tool., 1935*). Several authors have later confirmed these results. *Cl. difficile* is commonly isolated from fecal samples from healthy infants, sometimes in as high number as 10^9 g/feces (*Ellis-Pegler et al, 1975*). Also, in healthy adults, a substantial number can be found, up to ($10^{7.5}$ g/feces) (*Ellis-Pegler et al, 1975*). After a single dose of 2 g of cefoperazone, *Cl. difficile* was found in feces from 44% of volunteers investigated (*Privitera et al, 1991*). Thus, *Cl. difficile* is a commonly occurring member of the intestinal microflora in infants and adults.

Over the years, some reports have been published about the physiological influences of *Cl. difficile* in GF animals. In 1962 Skelly et al (1962) reported that *Cl. difficile* decreases the cecum size in mono-associated mice. Later on, Wiseman and Gordon (1965), found a transient reduction of cecum contents after contamination of GF animals either with *Cl. difficile* or *Salmonella typhimurium*. Gustafsson et al (1970) reported that *Cl. difficile* decreased cecum size as well as influenced upon intestinal motility in mono-associated rats. Høverstad et al (1985) reported that *Cl.difficile* give rise to several short chain fatty acids in mono-associated mice.

However, in 1978, *Cl. difficile* was recognized as a major cause of severe colitis following antibiotic therapy (*Bartlett et al, 1978; Larson et al, 1978; Borriello 1984*). Since then, causal relationships between presence of *Cl. difficile* and post antibiotic diarrhea have been studied in a vast number of investigations (*Hove et al, 1996; Aronsson et al, 1981*). It is also known that *Cl. difficile* can produce several toxins, especially enterotoxin A and cytotoxin B. The leading hypothesis for the harmful effect is that, following disturbances in the normal gut flora especially after usage of some antibiotics, *Cl. difficile* will proliferate in the intestinal tract, produce toxins that lead to colitis and diarrhea i.e., symptoms and signs that are caused by presence of both the bacteria and its toxins.

The host

Structure of small and large intestine

Small intestine: The small intestine is considered in terms of three regions, the duodenum, jejunum and ileum, but the distinction between these regions is not sharply defined. The surface of the intestinal mucosa projects into the lumen with numerous villi and at the base of these villi there are crypts (*Bloom and Fawcett., 1962; Potten and Loeffler., 1990*). Crypts supply cells to the villi, and several crypts surround the base of each villus; the number varies along the proximal-distal axis of the small intestine and also with age (*Falk et al, 1998; Cheng and Bjerknes., 1982; Wright and Alison., 1984*). As an example, in the mouse small intestine the crypts per villus ratio is 6-10 and in humans about 6 (*Potten 1992*). The crypt epithelium is composed of;

1. Undifferentiated cells including multipotent stem cells (*Bjerknes and Cheng., 1981; Cheng, 1974; Cheng and Leblond., 1974*) that are actively proliferating and involved in mitosis.
2. Differentiating cells, i e., the immediate descendants from the stem cells. These cells undergo migratory-dependent differentiation to give rise to at least six differentiated cell types found in the crypts and villi: a) mucus secreting goblet cells; b) a variety of endocrine epithelial cells; c) rare caveolated or tuft cells; d) Paneth cells with large secretory granules; e) a large number of absorptive cells, called enterocytes, and f) M cells overlying the Peyer's patches.
3. Functionally active cells, as maturated Paneth cells.

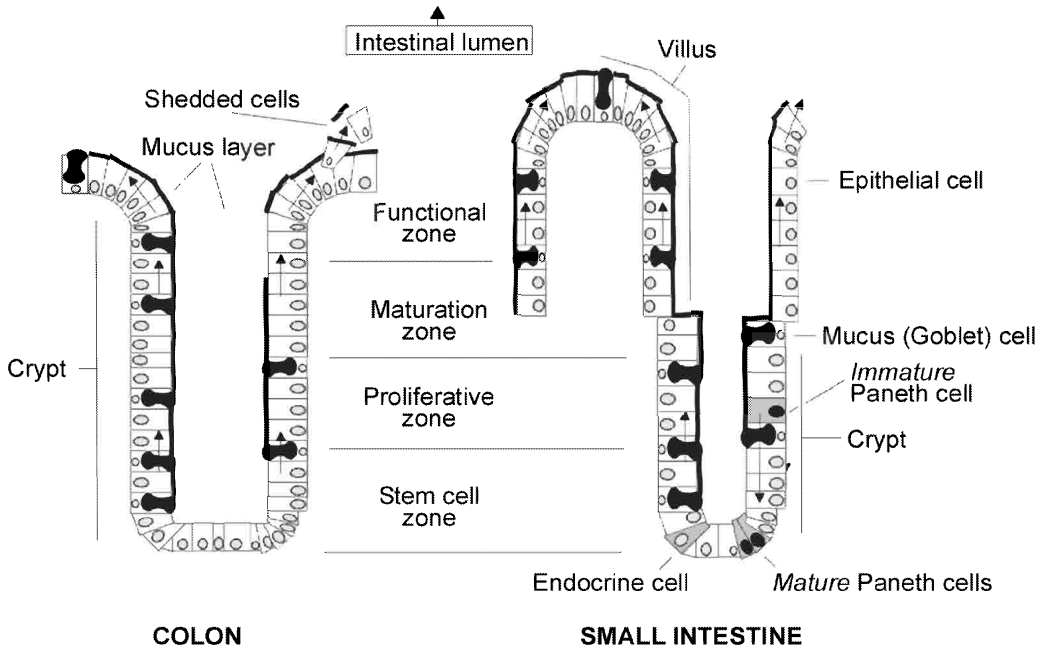

Figure 1. Diagram showing the structures, cell types, and relationship of the crypt and villus in the small intestine.

Large intestine: The colon has a flat epithelial surface. This surface is supplied from several tubular shaped crypts and the cells are extruded directly from the flat surface into the lumen. Four types of cells originate from the stem cells, which are located in the bottom of the crypts. These are; a) undifferentiated cells, which then differentiate into either, b) columnar absorptive cells c) goblet or mucus cells or d) enteroendocrine cells.

Cell kinetic aspects

Organisation of cell proliferation in the intestinal tract

In the intestinal tract, the epithelium is usually divided into proliferation and functional compartments. Cells are born in the proliferative compartment and migrate into the functional compartment. In principal, epithelial cell lineages arising from the multipotent stem cells are differentiating during an upward migration from the crypt to an associated villus. The major cell types in the villi include columnar absorptive enterocytes (by far the most dominating cell type in the villi), mucus-producing goblet cells, and enteroendocrine cells (*Leblond et al, 1948; Cairnie at al, 1965*). Between proliferative and functional zones a transitional zone can be identified where cells are loosing their proliferative capacities and undergo maturation (*Potten and Loeffler., 1990*). Thus, in the small intestine, proliferation, maturation, and functional compartments are found in the crypts while the villi epithelium

are functional compartments only. In the colon, proliferating and maturating cells are found in the lower part and functional cells are located in the upper part of the crypts.

Cell renewal in the small intestine

The small intestine is an organ in which the rate of cell turnover is one of the highest in the body (*Bertalanffy and Lau., 1962*). The epithelium covering the villi has long been recognised as part of a cell renewal system in which there is rapid and continuous replacement within the crypts (*Leblond and Walker., 1956*). The basic renewal system consists of the dividing cells of the crypts, which give rise to the columnar and goblet cells. The proliferative cells of the crypts can be labelled with radioactive thymidine that will label interphase cells, which are in the synthesis phase (vide infra). Another method is to use vincristine, which inhibits the microtubule organisation in the mitotic phase. The distribution of labelled cells or mitotic indices is as a function of the position of cells along the length of the crypt. The normal rate of cell production has been estimated to be in the order of 10^6 cells per 5 min in the mouse small intestine (*Hagmann et al, 1970 & 1971*). This means that a 25 g mouse will renew a mass of intestinal epithelial cells equal to its body weight every 4 month. The rate of migration of the epithelial sheet over the villous surfaces is such that a complete replacement of the intestinal lining is accomplished in approximately 2 to 3 days.

Phases of the cell cycle

The division cycle of most cells consists of four coordinated processes: cell growth, DNA replication, distribution of the duplicated chromosomes to future daughter cells, and cell division. The M phase of the cycle corresponds to mitosis. This phase is followed by the G1 phase (gap 1), which corresponds to the interval (gap) between mitosis and initiation of DNA replication. G1 phase is followed by S phase (synthesis), during which DNA replication takes place.

The completion of DNA synthesis is followed by the G2 phase (gap 2); during which cell growth continues and proteins are synthesised in preparation for mitosis. The duration of these cell cycle phases varies in different species and different kinds of cells. The total cell cycle time in the small intestine varies also considerably between species. However, the cell cycle time in the mice small intestine is usually 10-18 hrs and in large intestine is usually 15-36 hrs (*Potten 1992; Falk et al, 1998*). Various cell labelling methods can be used to study the length of different stages of the cell cycle. Since long, vincristine has been used to

block mitosis and is found especially suitable to follow cell kinetic parameters in rapidly dividing cell samples, as in the GI tract (*Wright et al, 1972*).

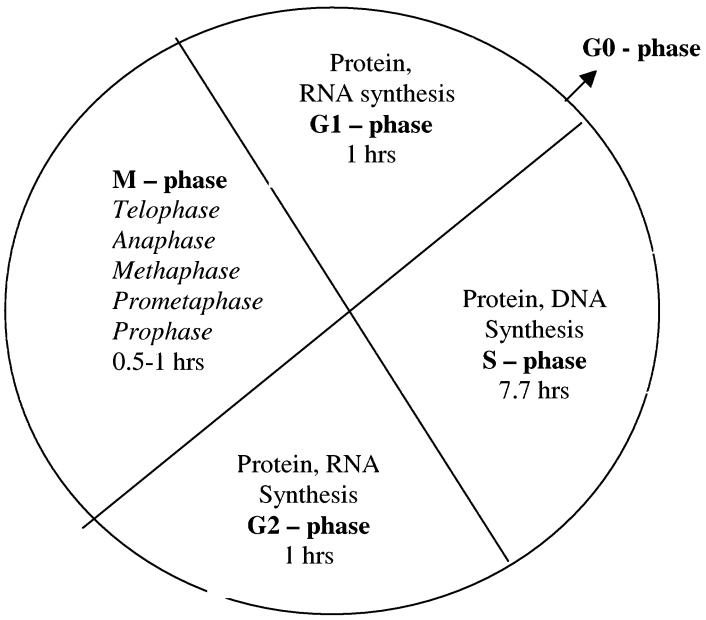

Figure 2: Different cell cycle phases in mice

Influence of some exogenous and endogenous factors on mucosa and cell regulation

The cell cycle of most dividing cells in mammals can be modified by a wide array of exogenous and endogenous factors.

In the GI tract mucosa, exogenous factors, such as dietary components (*Clarke 1975; Edwards et al, 1992*), and microbial products, including SCFAs (*Lupton and Kurtz., 1993; Mathers and Kennard., 1993*), are claimed to influence cell proliferation and differentiation. Fasting and refeeding (*Altmann 1972; Al-Dewachi et al, 1975; Goodlad et al, 1988*) and circadian variations (*Al-Dewachi et al, 1975; Hamilton 1979*) may also affect the cell proliferation rate.

Intraluminal factors such as pancreatic-biliary secretions modify the mucosal structure and function (*Altmann 1971*). Several endogenous peptides (*Tutton 1974; Bloom and Polak., 1982; Bataille 1989*) are also known to modulate cell proliferation in the GI mucosa. Apoptosis i.e., the ability of cells to undergo programmed cell death may facilitate the high turnover of cells in the intestinal epithelium (*Grossmann et al, 1998*). Comparative studies

in Conv and GF rats support a hypothesis of a feedback control by the functional villus cells on the regulation of the cell proliferation and maturation in duodenal crypts (*Galjaard et al, 1972*). Both age (*Lesher et al, 1961*) and endogenous factors, as testosterone (*Wright et al, 1972*) may also influence upon cell kinetics parameters in the GI tract.

Rats and mice as models for cell kinetic studies

Body weight pattern and some physiological parameters

Rats and mice have been selectively bred for different research purposes for many hundred generations. Laboratory rats are generally more docile and less aggressive towards members of their own species than mice are. In table 2 is shown the body weight of the animals used in the present studies. As the table shows, males weigh more than female counterparts by one exception (young Conv female rats weigh more than male). Weaning of the rats and mice is 28-30 days. Rats and mice are sexually mature at the age of 11-13 and 9-10 weeks, respectively, and life span for rats and mice is 3-4 and 2-3 years, respectively.

Table 2: General and specific physiological data of NMRI/KI mice and AGUS rats.

	Mice (NMRI-KI)	**Rats (AGUS)**
Weight (g)		
Young (35 days)		
Female	GF 20.3±1.7 Conv 19.9±1.2	GF 71.3±3.9 Conv 73.7±6.2
Male	GF 22.0±0.6 Conv 23.7±2.5	GF 76.2±1.4 Conv 67.9±1.7
Old (rats 190±60 and mice 184±16]		
Female	GF 24.3±2.3 Conv 24.2±2.1	GF 238.7±19.6 Conv 232.0±12.7
Male	GF 29.2±1.3 Conv 31.3±1.9	GF 354.0±20.8 Conv 349.5±13.8
Puberty (weeks)	***Female*** 5 ***Male*** -	***Female*** 6-8 ***Male*** -
Breeding age (weeks)	***Female*** 9-10 **Male** 9-10	***Female*** 11-13 ***Male*** 11-13
Weaning age (weeks)	4	4

Intestinal cell kinetic parameters in rats and mice

Table 3 summarises some previous data concerning the intestinal morphology in Conv and GF rats and mice (*Gordon 1960; Gordon and Pesti., 1971; Thompson and Trexler., 1971*). In GF animals, the wall of the small intestine is thinner, less well hydrated and reduced in weight. An absence of intestinal flora is generally assumed to reduce epithelial renewal processes thereby influencing upon the mitotic index and the generation cycle (*Abrams et al., 1963; Lesher et al., 1964; Cook and Bird., 1973; Guenet et al., 1970*). In both mice (*Abrams et al., 1963*) and rats (*Meslin et al., 1974*) villi of the distal small intestine seems to be shorter in the GF animals, and villi in the duodenum seems to be longer (*Meslin et al, 1974; Lesher et al, 1964*). The epithelial cells covering the villi were more uniform in the absence of a normal flora, and the microvilli were longer (*Abrams et al, 1963*). Somewhat surprisingly, it has been claimed that the total surface area of the small intestine is 30% less in GF rodents than in their Conv counterparts (*Gordon and Bruckner-Kardoss., 1961*).

Before drawing any further conclusion, it has to be mentioned that in most previous studies, information about age and gender of the experimental animals is either lacking or mentioned fragmentarily.

Table 3: Number of cells in the crypts and villi of GF and Conv animals at different sites along the intestinal tract.

Site (Species)	**Status**	**Number of crypt cells**	**Number of villus cells**	**Investigators**
Ileum (M)	GF	13.2±0.1	36.0±0.8	Abrams et al (1963)
	Conv	17.3±0.3	40.4±0.6	
Ileum (M)	GF	22.0±2.6	63.1±9.0	Matsuzawa and Wilson
	Conv	33.5±4.2	82.2±16.9	(1965)
Duodenum (R)	Conv	34.4±1.7	77.6±5.1	Uribe et al (1997)
Jejunum(15cm)(R)		27.7±2.8	76.4±4.3	
Jejunum(60cm)(R)		30.4±2.6	64.6±3.0	
Duodenum(R)	GF	32	105	Galjaard et al (1972)
	Conv	31	76	
Duodenum (R)	GF	20	93	Meslin et al (1974)
	Conv	22	75	
Jejunum (R)	GF	19	75	
	Conv	21	70	
Ileum (R)	GF	17	45	
	Conv	21	49	
Jejunum (15cm) (R)	GF	29.7±2.6	88.2±5.1	Uribe et al (1990)
	Conv	30.3±1.2	79.5±3.1	
Ileum (R)	GF	28.6±2.3	51.2±2.9	Alam et al (1994)
	Conv	28.5±1.5	43.0±2.9	

(M) = Mice, (R) = Rats

AIM OF THE STUDIES

i. Standardisation of experimental conditions for studies of influence of gender, age and microbial flora on intestinal mucosal cell proliferation and morphology in rats and mice (paper I and II).

ii. To study the influence of a symbiotic strain, i.e. *Lactobacillus rhamnosus* GG on the intestinal mucosal cell proliferation and morphology in mono-associated rats (paper III).

iii. To study the influence of a potential pathogenic strain i.e. toxin positive *Clostridium difficile* on the intestinal mucosal cell proliferation in mono-associated rats (paper IV).

iv. To study influence of a commensal i.e. a toxin negative *Clostridium difficile* on the mucosal cell proliferation in mono-associated rats (paper IV).

MATERIAL AND METHODS

Animals

The AGUS strain (*Festing 1993*) is the main rat strain at the laboratory and it has been kept GF and inbred for close to 90 generations. The breeding has followed brother-sister mating with maximum of 3 litters per generation (Paper I, III, IV). NMRI/KI mice have been kept at the laboratory for more than 35 generations (Paper II). The mouse breeding has also followed brother-sister mating in up to 6 litters per generation. In order to avoid a genetic drift, GF rats/mice are conventionalized (the animals are inoculated with homogenate feces from Conv animals) every 3rd generation and their offsprings are used as Conv counterparts. At regular intervals, the Conv animals are checked for presence of a functionally active intestinal microflora (*Midtvedt et al, 1985*) as well as checked for absence of pathogens, according to an internationally established standard (*FELASA 2001*). The GF animals are checked weekly for the germfree status by inoculating fecal samples in TGB (Tryptose, DIFCO Laboratories Detroit MI, USA and Glucose Broth, Mallinckrodt Chemical Works, St. Louis, MO, USA) and RCM (Reinforced Clostridial Medium, MERCK, Darmstadt, Germany) aerobically, and THM (THioglycollate Medium, DIFCO) and ACM (AC Medium, DIFICO) anaerobically, and these are inoculated at both 20°C and 37°C, respectively. All samples are checked for growth regularly for up to two weeks.

All animals had free access to a steam-sterilized (*Gustafsson 1959*) standard rodent diet (R36, Lactamin, Vadstena, Sweden) and water. Artificial light was available between 6 a.m. to 6 p.m., temperature (24±2°C) and humidity (55%±10%).

i. Standardisation of experimental conditions (paper I and II)

Rats: Twenty-three GF and twenty-four Conv AGUS rats, gender and age matched, were divided into groups of 6 animals with one exception: 5 animals only in the group of male old GF rats. The age of the young rats was 35 days and the old rats 190 ± 60 days. (Paper I)

Mice: Twenty-four GF and twenty-four Conv NMRI/KI mice, gender and age matched, were divided into groups of 6 animals each. The young mice were 35 days old and the old mice were 184±16 days old. (Paper II)

ii. The influence of LGG (paper III)

Sixteen GF and five Conv AGUS rats were used in this study. The rats were 35 days of age when included in the study. Ten GF animals were mono-associated with LGG and six GF

rats were used as controls. The mono-associated rats were divided into two groups of 5 rats in each which were kept mono-associated for 3 and 21 days, respectively.

iii. The influence of toxin positive *Cl. difficile* (paper IV)

Nineteen GF AGUS male rats, 35 days old were divided in three groups (6, 7, 6 in each) and then each group was mono-associated with a toxic producing *Cl. difficile* (strain 79-685) for 3, 7 and 21 days respectively, in one isolator.

iv. The influence of toxin negative *Cl.difficile* (paper IV)

Another 17 GF AGUS rats were divided into three groups (6, 5, 6) and mono-associated with the toxin negative *Cl. difficile* strain CCUG 8884, for 3, 7 and 21 days, respectively.

Bacteria strains and growth conditions

Lactobacillus rhamnosus GG (ATCC 53103, Valio Ltd, Helsinki, Finland) was grown in MRS broth (de Man, Rogosa and Sharpe, MERCK, Darmstadt, Germany) anaerobically at 37°C for 24 hrs, before transferring into the isolator in a sealed ampoule (paper III).

Clostridium difficile, strain 79-685 was a gift from Prof P Bourlioux, Université Sud, Paris, France. This bacterium was highly virulent, producing toxin A and B. A non-virulent *Cl. difficile* strain CCUG 37785 was obtained from Culture Collection, University of Gothenburg, Sweden. These two strains were grown in ACM broth plus 0.1% agar (DIFCO), incubated anaerobically at 37°C for 2 days, before taking into respective isolator.

Mono-association

After incubation, cultures of 10 ml were dispensed into sterile ampoules, which were heat sealed. The external surfaces were sterilized with chromsulfuric acid and thereafter, the ampoules were transferred into respective isolator. Inside the isolator, the ampoules were broken and 0.5-1 ml (10^9 CFU/ml) of the content was given orally to each animal and the remaining content was spread on the fur and bedding material.

General procedures

Blocking of mitosis

Vincristine (Oncovin, Lilly S.A. Fegersheim, France) 1 mg/kg was injected intraperitoneally (ip) (*Frej et al, 1964; Tannock 1965; Uribe and Johansson., 1988*) starting at 10^{00} a.m. All animals were subjected to laparotomy following an injection of mebumal (25mg/kg) ip, exactly 4 hrs after the vincristine injection.

Preparation of specimens

Aliquots of two cm of tissue samples were taken from the duodenum, jejunum at 15 / 10 and 40 / 25 cm distal to the pyloric region in rats and mice, respectively, and from ileum 5 cm proximal to the ileo-cecal junction. In the large intestine, samples (1 cm) were also taken from the cecum at its base and from the colon (2 to 3) / (1 to 2), (5 to 6) / (3 to 4) and (8 to 9) / (5 to 6) cm from the cecum in rats and mice, respectively. Each specimen was placed on a micropore filter (0.2 μm, Schleicher & Scuell, Dassel, Germany), cut open along its longitudinal axis to obtain a good orientation of crypts and villi and fixed for 3 hrs in Carnoy´s solution. Thereafter, all specimens were placed in 70% ethanol for at least 20 hrs before processing to paraffin embedding. Thereafter, 3 μm thick sections were taken from each specimens 54-100 μm apart from each other and stained with hematoxylin and eosin.

Microscopic evaluation

All sections were coded and examined in a blind fashion (by one investigator) under light microscope (X200 Leica DM LS, Wetzlar, Germany).

Mitotic index (MI)

The MI is the percentage of cells in the metaphase and is calculated by the formula

MI% = Nm/Nt X 100, where the Nm is the numbers of mitotic cells and Nt is the total number of cells in the left column of the crypts. The MI was calculated in 30 well oriented crypts (I, II, III, IV).

Number of epithelial cells in crypts and villi

The total number of cells was counted in a similar manner to MI in 30 well-oriented consecutive crypts in the small intestine (IV) and colon (I, II, III) and in the villi of the small intestine, respectively (I, II, III, IV).

Depth of crypts, height of villi and crypt/villus ratio

In the small intestine, the depth of 20 well oriented crypts were measured from the base of the crypt to the crypt-villus junction and the heights of 20 villi were measured from the crypt-villus junction to the tip of the villi by using a micrometer in the ocular eye-piece (magnification x 100) (I, II, III, IV). In a similar way the depth of colonic crypts was measured from the base of the crypt to the flat margin of the colonic mucosa (I, II, III).

Further, in the small intestine the total number of crypts and villi present in 1 mm of mucosa were counted by an ocular eye-piece micrometer placed in the ocular eye-piece (magnification x 100) to estimate the crypt/villus ratio (I, II, III).

Growth fraction (GrF)

The GrF is the portion of the cell populations, which are actively engaged in proliferation. To correct for differences due to crypts of unequal size, a normalised crypt were used (*Cairnie and Bentley., 1967*). The cut-off position was estimated by an intersection of the half peak position with a regression curve (*Uribe and Johansson C., 1988*) (I).

Toxin measurements

Cytotoxicity assays: Appropriate amounts of the small and large intestine content from each rat were suspended in 0.5-1.0 ml of 1:10 diluted sample buffer supplied with the *Cl.difficile* toxin A/B EIA kit (RIDASCREEN® Clostridium difficile Toxin A/B, R-Biopharm GmbH, Darmstadt, Germany). After extensive vortexing and centrifugation (13000 rpm), aliquots of the supernatants were diluted and toxin measurements were performed as described in the EIA kit instructions. One U of toxin (including both toxin A and B) was defined as one absorbance (A_{405}) per ml original suspension, and finally recalculated as U/g intestinal material. The cut-off level of toxin was set to 100 U/g (IV).

Statistical analysis

Results are given as the mean ± standard deviation (SD). The analysis of variance (ANOVA) was used to evaluate the differences between the groups. The significance level was $p<0.05$.

RESULTS AND DISCUSSION

As is evident from ''Materials and Methods'', all four studies have the same experimental design. The focus will be on the importance of a strict standardisation of experimental conditions when studying intestinal cell kinetic and morphology, especially in relation to microbial status of the host.

Methodological aspects

Vincristine

It is well established that '' no single technique can give a comprehensive picture of the cell kinetics of a tissue'' (*Uribe 1988*). The proliferative activity of a cell population is usually determined by either recording the fraction of cells synthesing DNA or recording the number of mitotic figures. In the present study, we employed the second approach, i.e. we gave a metaphase blocker in order to calculate the percentage of cells that had reached the metaphase during our observation period. According to Tannock (*Tannock 1965*), a suitable metaphase blocker should posses the following characteristics:

a) There must be an optimum dose at which it arrests all metaphases in the tissue under study over a certain period of time.
b) The arrested metaphases should not degenerate before the tissue is fixed.
c) The mitotic arrested properties should not be highly sensitive to the dose.
d) There should be no effect on interphase cells.

Over the years, vincristine has become the most commonly used metaphase blocker in experimental intestinal cell kinetic studies. Following injection, vincristine goes rapidly into the intracellular compartment and blocks the metaphase by acting as a microtubule inhibitor. In the present studies, all animals received 1 mg/kg of vincristine and that is supposed to be an optimum dose (*Tannock 1965; Uribe 1988; Alam 1995*). Additionally, it has been shown that 3.0 mg/kg vincristine did not increase the number of metaphases in the cornea or rectum of the mice over that recorded for a dose of 1.0 mg/kg vincristine (*Burns 1984*).

When employing a metaphase blocking technique, it is of importance to carry out a linearity study. Most probably, the time span for metaphase blocking may be tissue dependent. In tissues not lining a lumen filled with microbes, the observation period can be as long as 12 hrs as in bone marrow studies (*Frei et al., 1964*) and 10 hrs in implanted tumour studies (*Smith et al, 1974*). In intestinal cell kinetic studies, the observation periods are usually 2 to 4 hrs (*Tannock 1965; Uribe 1988; Alam 1995*). In some preliminary

experiments, we investigated the linearity following injection of vincristine. As is evident from table 5, linearity was obtained up to 4 hrs, without any alteration in the number of crypt cells. Consequently, an observation period of 4 hrs was used in the present studies. In intestinal cell kinetic studies, it is generally assumed that an extension of the accumulation period beyond 4 hrs may give rise to estimation errors due to degeneration of mitotic figures (*Uribe 1988, Aherne and Camplejohn., 1972*). However, whether and to what extent this degeneration parallel each other under GF and Conv conditions has, to the best of our knowledge, not been investigated.

Table 5: Mitotic index ± SD and total number of crypt cells ± SD, in duodenum, when applying different arrest times in Conv female old mice.

Groups / Arrest time	Mitotic index	Crypt cells
Blocked for 2 hrs	10.3 ± 1.0	15.3 ± 0.6
Blocked for 3 hrs	14.3 ± 1.4	15.7 ± 0.3
Blocked for 4 hrs	19.4 ± 2.3	15.6 ± 0.4

Fasting time

It is a well established fact that dietary manipulation, including fasting, in several ways may influence on intestinal cell proliferation. A number of studies (*Hagmann and Stragand., 1977*) have dealt with starvation effects on intestinal cell proliferation in rats and mice, and some major alterations will be outlined. During the fasting period, the proliferative compartments in small intestinal crypts will decline significantly, accomplished by a reduction in total crypt cellularity.

The first observation was made after 24 hrs of fasting. In the colon, however, fasting had no effect on the size of the proliferative zone or on the total crypt cellularity. In both compartments they found a reduced production of new cells and an increase in the total cell cycle time, mostly due to an increase in GI tract.

Obviously, the length of the fasting period prior to an intestinal cell kinetic experiment may have implication on the actual kinetic profile in that organ. As mentioned in paper I (Table V), previous investigations on comparative cell kinetic studies in rats may have paid too little attention to the possible influence of the fasting time. The fasting time was either not stated, reduced to zero (free access to food), or could be up to 96 hrs.

With reference to our own studies, it is worth mentioning, that before we settled a fasting period of 2.30 hrs, we tried to take into account some basic physiological data on laboratory rodents. It is well known that rats (probably more than mice) are ''night animals'', i.e., they eat preferably in the dark. Therefore, when taken away the food in the evening before start of the experiment, the actual fasting time will be substantially longer than around 12 hrs. The social ranking within the group of animals present in one cage may also influence upon their access to food.

However, in all cases, all animals will eat during the night. Therefore, setting the fasting time as short as 2.30 hrs and starting by daylight, the actual fasting time for each animal may vary from 2.30 to around 12 hrs. As far as we are aware of, a fasting period of 12 hrs or a less may have little, if any, influences upon intestinal cell kinetics and morphology.

Aspects on species and species differences

As mentioned earlier, one major objective behind the present investigation was to standardise the experimental design. Our AGUS rat strain has been kept in the laboratory under GF conditions for more than 100 generations – and had – at the start of this investigation – been inbred for around 73-75 generations. Similarly, the NMRI/KI mice have been kept for more than 50 generations and had been inbred for around 37-40 generations when the experiments were performed. In order to avoid a genetic drift between the GF and Conv animals, some couples of GF animals are brought out from the isolator every 3rd generation. These animals are heavily inoculated with a homogenised cecal flora from Conv animals of the same species. The off-springs of these ex-GF conventionalised animals serve as our main stock of Conv animals for the next 3 generations.

In addition to this very strict and similar breeding regimen, it should be mentioned that all Conv animals were kept in the same animal room. All animals regardless of microbial status received the same steam sterilised diet and they were raised on the same bedding, and, as stated in paper I and II, the outer physical conditions were the same. It seems reasonable to assume that all these precautions that have been taken into account are allowing us to make some comparisons and speculations on intestinal cell kinetics and morphology between rats and mice (Table 6).

The first statement to be made is that there is a high degree of similarity between the values of MI in these two species, being high in the upper parts of the small intestine and low in the colon. A second similarity is the crypt/villus ratio, being at really the same levels throughout the small intestine.

A second statement to be made that when working with the GAC/MAC concept, it is of paramount importance to have reliable and constant baselines. Intestinal cell kinetic is a parameter that can be - and is - influenced upon by several factors. Therefore, if you want to study the influence of one factor, i.e. microbial status, other influencing factors, as species, strain, animal age, sex, diet, fasting time etc. etc, should be equalized as far as possible. As show in paper I (table V), previous investigations of intestinal cell kinetics and morphology in GF and Conv rats are hampered with a very variable experimental design making a more close comparison with our results somewhat uncertain and probably also misleading. Therefore, instead of commenting upon possible weaknesses in previous investigations it seems far more proper to underline the importance of a strict standardisation in future investigations.

Thus, by emphasising a strict standardisation, it is not contradictory that MI in some intestinal compartments can be far higher in GF as compared to Conv animals, even if it is a general phenomenon that presence of microbes may trigger the MI. As discussed in paper I, GF animals, especially young male rats, need more food than their Conv counterparts. More food means a need for a greater area of absorption, i.e. more cells - and a higher MI.

The third statement that can be made is, that establishment of a bacterial strain as a mono-associate seems to initiate a rapid trigging of MI, irrespectively of whether it is a presumed probiont (III), pathogen (IV), or commensal organism (IV). Whether or not this observation holds true for a greater number of strains and species remains to be further investigated.

The forth statement that can be made is that following this rapid initial increase in MI in mono-associated animals, the MI's will slowly switch to Conv values. The low number of bacterial strains investigated in this thesis do not allow further conclusions to be drawn, but it is tempting to forecast that each species - or strain - may have its own MI influencing profile. Factors influencing upon this profile might be several, as a compartmentalised establishment, capability to break down higher carbohydrates, mucin, nitrogen-containing compounds etc. The continuing compartmentalised cross-talk between the mono-associated strain and its host will set a new compartmentalised level of MI's.

The fifth statement that can be made is that a potential pathogenic microbe, i.e. a toxin producing *Cl. difficile* may have a marked influence on intestinal cell kinetics without leading to the death of its host. However, our results do not exclude that *Cl. difficile* might be lethal, even in rats. The cross-talk between *Cl. difficile* and its host can be described as a continuum of interactions that may span symbiosis (improvement of motility etc), commensalism (conventionalisation of MI's) to pathogenicity (very marked reduction of MI's). Our results indicate that these market alterations in MI's are initiated by production of toxins. The lethal effect(s) of *Cl. difficile* might be caused by *Cl. difficile* itself or presence of microbes that might be able to attach the epithelial line due to its markedly reduced upward flow. However, studies of these two mechanisms were not the scope for thesis.

Table 6: Number of significant differences due to gender, age and microbial status on intestinal cell kinetic and morphology parameters in rats and mice, in paper I (Table III) and in paper II (Table VI).

Cell kinetic Parameters	Small intestine Mice	Large intestine Mice	Small intestine Rats	Large intestine Rats
Mitotic Index	**0 4 2**	**0 0 8**	**4 0 2**	**3 4 1**
Crypt Cells	**1 6 6**	**2 2 9**	**1 9 8**	**2 7 8**
Crypt Depth	**0 3 7**	**3 2 16**	**7 9 4**	**2 2 5**
Villus Cells	**2 3 7**	_	**2 8 8**	_
Villus Height	**1 2 8**	_	**4 8 5**	_
Crypt/Villus ratio	**2 1 2**	_	**1 3 2**	_
Total number of significance differences	Gender (6) Age (19) Microbial status (32)	Gender (5) Age (4) Microbial status (33)	Gender (19) Age (37) Microbial status (29)	Gender (7) Age (13) Microbial status (14)

Conclusion and perspectives

The intestinal one-layer epithelial border is the host's major line of defence and the continuos flow of new cells entering that defence line is the major homeostatic regulatory principle. Obviously, increased knowledge of the basic mechanisms behind this regulation is mandatory when trying to make therapeutic improvements in intestinal disorders.

A reduction in the intestinal absorptive area is a sad ''side–effect'' of some chronic diseases, as AIDS and some mal-absorption syndromes. An up-regulation of the production of enterocytes might tend to enlarge the absorptive area – thereby, improving the clinical state of the patient.

Mucosal damages are important parts of several intestinal pathophysiological conditions, as antibiotic–associated diarrhea and inflammatory bowel diseases. An increased production of enterocytes might speed up the reparative events, i.e., the mucosal lining will be re-constructed. Even a more pronounced dys-balance in production/elimination of intestinal cells, as in carcinomas, might be a future goal for these regulatory principles.

Protocols for studies of some of these aspects are in progress. In all these protocols, use of gnotobiotic models are the corner-stones in the investigations. In such experiments, the very complex and intricate intestinal ecosystem is reduced to a simplified model in which a single – or a defined number of – bacterial strains can be studied for their capabilities of initiating or continuing cross-talks with the host under physiological as well as pathophysiological conditions.

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