Maturation of the rat small intestine at weaning: changes in epithelial cell kinetics, bacterial flora, and mucosal immune activity

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SUMMARY

The relationship between maturation of the small intestine and change in mucosal immune activity was examined in the DA rat during the weaning period from 12 to 30 days. Two stages of jejunal maturation were observed: an initial stage of morphological development and crypt proliferation (days 12 to 22), followed by a period of stabilisation (days 24 to 30). By day 22 of the initial phase, villi increased principally in width but not in length, crypt length increased, and crypt cell production rate increased from 0.5 (day 12) to 11.1 (day 22) cells/crypt/hour. Various measures of mucosal immune activity showed a biphasic response. Up to days 20 to 22, the weight of the mesenteric lymph node increased seven-fold (p<0.0001), counts of jejunal eosinophils and goblet cells increased 3- (p<0.0001) and 19-fold (p<0.0001) respectively, and mean serum rat mucosal mast cell protease II, released from mucosal mast cells, increased from 24 (day 12) to 313 (day 22) ng/ml (p<0.0001). After day 22, mesenteric lymph node weight stabilised, eosinophil count stabilised and goblet cells decreased, serum rat mucosal mast cell protease II decreased three-fold (p<0.0001), and mean jejunal count of intraepithelial lymphocytes increased from 26 (day 22) to 54 (day 24) cells per mm of muscularis mucosa (p<0.0001), before stabilising. These results demonstrated a close association between maturation of the small intestine and change in activity of the mucosal immune system.

The small intestine in the rat undergoes a process of development and maturation that is associated with weaning; its weight increases from 15 days of age, and this is associated with lengthening of intestinal crypts and with increased cell proliferation. Interestingly, suckling and germ free animals have fewer intestinal lymphoid cells than adult animals, and their intestinal crypts are smaller and less active in proliferation. As cell mediated responses cause crypt lengthening and increased crypt cell production rate (CCPR) in enteropathies, it is possible that the effect of bacterial flora on mucosal morphology and epithelial cell proliferation is mediated by antigen driven activation of local T cells. There have been no studies, however, to relate mucosal immune activity to change in bacterial flora and gut maturation during weaning.

As it is difficult to directly measure cellular immune activity in the gut, surrogate measures need to be used such as jejunal count of intraepithelial lymphocytes (IEL) and crypt lengthening. These are characteristic of a delayed type hypersensitivity reaction that accompanies graft versus host reaction (by definition cell mediated), intestinal allograft rejection, and protein fed antigen immunisation. These are particularly useful when the putative antigenic stimulus is unknown. Further measures are also available in the rat, as expansion of mucosal mast cells (MMC) and goblet cells are known to be T cell mediated, while systemic release of rat mucosal mast cell protease II (RMCPII) from MMC serves as an activation marker that is raised during graft versus host reaction, although RMCPII is also released by an IgE mediated mechanism during anaphylaxis. Eosinophils have also been shown to be under T cell influence systemically, although a similar effect has not been studied in the small intestine. Jejunal counts of IEL, MMC, goblet cells, and eosinophils, and
serum RMCPII estimation were therefore used as surrogate measures of mucosal cell mediated immune activity.

The aim of this study was to relate development of the small intestine from 12 to 30 days of age to changes in mucosal immune activity in the rat. This period was chosen to encompass pre- and post-weaning events. Intestinal maturation was measured by intestinal morphology, epithelial cell kinetics, and activity of disaccharidase and alkaline phosphatase enzymes. We also examined colonisation of the small intestine by carrying out Gram stains and culture on gut washings.

Methods

RATS

Groups of six DA rat pups aged 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 days were selected from litters of approximately the same size, although runted animals were excluded. Guidelines for animal experimentation of the National Health and Medical Research Council of Australia were followed.

PROCEDURES ON DAY OF KILLING

A group of pups was anaesthetised with ether and injected ip with vincristine (1 mg/kg). This initial injection was given at approximately 0900 h. Animals were reanaesthetised at progressive time intervals after injection and exsanguinated by decapitation or from the axillary artery. Blood was collected for serum RMCPII estimation. The small intestine was removed under aseptic conditions and the length recorded after gently stretching the intestine at a 30 degree angle. Three 0·5 cm jejunal segments were taken (the first at 10 cm from the pylorus and the other two in succession proximally) and orientated onto cardboard. One sample was fixed in Clarke’s fixative (75% ethanol:25% acetic acid, v/v) with shaking for microdissection (the exact elapsed time was recorded since vincristine injection), another in Carnoy’s fixative for MMC counts, and the third in freshly prepared 4% paraformaldehyde/phosphate buffered saline (pH 7·4). Three cm of adjacent proximal intestine was frozen for disaccharidase assays. The mesenteric lymph node (MLN) and spleen were removed and weighed. A further length of jejunum was removed at 10 to 20 cm from the pylorus under aseptic conditions and used to prepare gut washings for bacterial counts.

MESENTERIC LYMPH NODE AND SPLEEN WEIGHTS

The MLN of each animal was carefully dissected from the root of the mesentry of each animal and weighed. Spleens were removed and weighed.

BACTERIAL COUNT OF THE JEJUNUM

Using a sterile 19 gauge hypodermic needle and a 10 ml plastic syringe, 5 ml sterile 0·15 M sodium chloride was washed through 10 cm of jejunum, followed by 5 ml air, and collected from the distal end into a sterile Universal container. A drop of residual gut washing was examined as a wet film for protozoa. Five millilitre gut washing (neat and 1/10 dilution) was poured onto a preweighed blood agar Petri dish for aerobic culture, and drained onto a similar blood agar Petri dish for anaerobic culture. Both Petri dishes were reweighed to determine the inoculating volume. Agar plates were incubated overnight (37°C) for aerobic culture and for 48 h for anaerobic culture. The total number of colonies was counted on each plate. As the inoculating volume of gut washing was known, bacterial count was expressed as organisms per ml of gut washing, as enumerated by colony forming units. After incubation, Gram stains were made from representative colonies on each plate. A small area of small intestine at 21 cm from the pylorus was opened and smeared onto a microscope slide for Gram stain of mucosal smears.

INTESTINAL MORPHOLOGY AND EPITHELIAL CELL KINETICS

Intestinal tissue was fixed in Clarke’s fixative overnight and stored in 70% ethanol. For microdissection, stored tissue was rehydrated, and hydrolysed for eight minutes in 1 M hydrochloric acid at 60°C before staining with Feulgen reagent (# 8542.88, Difco, Surrey, UK) for 40 minutes. Fragments of tissue were microdissected using a stereomicroscope, and examined after mounting in 45% acetic acid. Using a calibrated graticule, the length and maximal basal width of 15 villi, and length of 15 crypts were measured. The number of metaphases was counted in 15 crypts. This was used to calculate the CCPR from the rate of accumulation of metaphases after vincristine injection using a regression line of least squares estimate.12 The remaining Feulgen stained tissue was mounted in 45% acetic acid between two microscope slides and the ratio of the number of crypts to villi determined under microscopy using a square graticule.

JEJUNAL COUNTS OF EOSINOPHILS, IEL, GOBLET CELLS AND MMC

Intraepithelial lymphocyte goblet cells and MMC counts were carried out on jejunal tissue fixed in Carnoy’s fixative, while eosinophil counts were done on tissue fixed in 4% paraformaldehyde/phosphate buffered saline. Tissues were embedded in paraffin, and histological sections cut at 4 μm. Haematoxylin and eosin was used to stain IEL and goblet cells, Alcian blue (pH 0·6)/safranin was used to stain for
MMC, and carbol chromotrope was used to stain eosinophils. Cell counts were enumerated using a linear microscopic graticule (323 μm, ×25 objective lens) aligned along the muscularis mucosae of each sample. An average of 10 counts was obtained for each animal. All counts were expressed as cells per mm of muscularis mucosae.

**DETERMINATION OF SERUM RMCPII**

A solid phase antigen capture sandwich enzyme linked immunosorbent assay (ELISA) was used. The assay was developed by Dr J Huntley, Moreden Research Institute, Edinburgh, UK. Microtitre plates were coated overnight with sheep IgG anti-RMCPII (1 μg/ml in carbonate/bicarbonate buffer, pH 9-6). After washing, RMCPII standards (25-100 ng/ml) and dilutions of samples were added in duplicate for one to two hours. After incubation and washing, affinity purified sheep F(ab')2 anti-RMCPII peroxidase conjugate (1/4000) was added for one to two hours, and a colour reaction developed using o-phenylenediamine/hydrogen peroxide substrate. RMCPII concentration was read from the standard curve and expressed as ng/ml.

**DISACCHARIDASE AND ALKALINE PHOSPHATASE ASSAYS**

A 3 cm segment of small intestine was homogenised (1:9 w/v) in 0-15 M potassium chloride and centrifuged at 800 g for 15 minutes before freezing at −20°C for storage. Disaccharidase and alkaline phosphatase assays were modified to use microtitre 96-well ELISA plates and so that they could be read spectrophotometrically by an ELISA reader. For lactase (EC 3.2.1.23), sucrase (EC 3.2.1.48), and maltase assays, 50 μl homogenate was incubated (1/2, 1/4, 1/10) with the appropriate disaccharide substrate for 60 minutes. A colour reaction was developed with these samples (10 μl, 1/20 dilution), and with individual disaccharide blanks, as well as with a glucose standard curve (125-750 μM) using glucose oxidase reagent. Disaccharidase activity was expressed as μmol of disaccharide hydrolysed/min/g wet weight of jejunum. For the alkaline phosphatase (ED 3.1.3.1) assay, gut homogenate was diluted, added (25 μl, 1/100) to 100 μl p-nitrophenol phosphate substrate (0-15 M) on an ELISA plate, and incubated for 30 minutes before being read. Alkaline phosphatase activity was expressed as μmol of nitrophenol phosphate hydrolysed/minute/g wet weight of jejunum. Neither disaccharidase and alkaline phosphatase activities were related to protein to avoid assumptions about the relationship of enzyme activity to total protein.

**STATISTICAL ANALYSIS**

One way analysis of variance was used to test for significance difference of various group measures against time of weaning. Where necessary, individual paired comparisons were made using Peritz’ F test using a 95% experimentwise confidence interval. Where data displayed a skewed distribution, a log(x) transformation was done to normalise the distribution and stabilise the variance before significance testing. Crypt cell production rate was calculated from the least squares estimate of the linear regression of number of blocked metaphases with time after vincristine injection. Both Peritz’ F test and CCPR were computed using programs adapted for the Apple Macintosh computer.

**RESULTS**

**GENERAL FEATURES**

Rats pups opened their eyes after 18 days of age as they started to become less dependent on the dam rat. At the time of killing, milk was present in the gut of these pups, but decreased in volume and consistency by days 20–22. By day 22, formed faeces was present in the distal small intestine and large intestine.

**BODY WEIGHT AND INTESTINAL LENGTH**

Body weight and intestinal length are given in Table 1. Weaning was associated with an increase in these measures.

**SPLICE AND MESENTERIC LYMPH NODE WEIGHTS**

Spleen weight did not significantly increase over days 12 to 18 – for example, day 12< day 18, p=0.29, but increased after this time (Fig. 1). In contrast, MLN weight increased three-fold from days 12 to 18.
Maturation of the rat small intestine at weaning

Fig. 1  Development of the spleen and mesenteric lymph node during weaning in the DA rat from 12 days of age. Each age interval represents the mean (SD) of six rats.

(p<0.0001); it reached a maximum weight with a seven-fold increase by day 24 (v day 12, p<0.0001), and stabilised with no significant change to day 30 (Fig. 1). The ratio of MLN weight to spleen weight remained low before weaning, but increased to a peak on day 20, before falling to a value which remained higher than the preweaned ratio.

BACTERIAL COUNT OF GUT WASHING AND DIRECT MICROSCOPY

In preweaned animals, direct Gram stain and culture revealed only Gram positive bacilli, morphologically resembling Lactobacilli, and cocci. Five colony types of bacteria could be distinguished. During weaning, the total number of gut bacteria (colony forming units) decreased by 2 \text{log}_{10} \text{units} to reach a nadir at day 18 of age, and increased again by 2 \text{log}_{10} \text{units} to a plateau value by day 24. Gram negative organisms were first identified on day 18 by Gram stain of mucosal smears and by culture of gut washing. Approximately 16 different species of bacteria could be distinguished from about day 20, using such criteria as Gram staining and colony morphology. Occasional fungi were also seen on direct Gram stain and were grown on culture from day 22. No protozoa were observed on direct wet film examination of gut washing.

INTESTINAL MORPHOLOGY AND EPITHELIAL CELL KINETICS

Twelve day old rat pups had finger shaped villi. During weaning, the width of the villi increased as they became more leaf shaped. Intestinal crypts were small before weaning and lengthened during the period from about 16–18 days. Enterocytes of suckling animals contained lipid droplets up to 20–22 days. Quantitative morphological measurements are given in Table 1. Villus length increased slightly by day 24 (v day 12, p=0.025), but otherwise there was no significant alteration. Maximal basal width of villi and intestinal crypt length increased but in both cases the principal increase occurred up to day 24. Epithelial cell kinetics, measured by CCPR (Fig. 2), increased approximately 22-fold from days 12 to 30. An exponential increase in CCPR occurred up to day 24, whereas values fluctuated after this time before stabilising.

JEJUNAL COUNTS OF EOSINOPHILS, IEL, AND GOBLET CELLS

Counts of eosinophils, IEL, and goblet cells are given in Figure 3. Eosinophils began to increase after day 18 – for example, day 18 v 20, p<0.0001, and reached a 3-fold peak by day 24 (p<0.0001) before stabilising. On days 12–14, the majority of eosinophils were located in the lamina propria, usually around the basal portion of the villi or in the pericryptal region, but some eosinophils were seen in an intraepithelial position after day 16, and showed a distribution that was more uniform along the whole villus length. Intraepithelial lymphocytes increased by 50% from day 12 to day 22 (p=0.0032), by two-fold at day 24 (p<0.0001), and then remained stable until day 30. Goblet cells increased exponentially 19-fold during the weaning period until day 24, and decreased slightly until day 30. Thus, both eosinophils and
Fig. 3  Intestinal counts of goblet cells, eosinophils, and intraepithelial lymphocytes in DA rats during weaning from 12 days of age. Each age interval has the mean±SD of six rats.

goblet cells increased up to day 24, and later either stabilised or declined, while IEL had an abrupt and delayed two-fold rise to a more or less stable value at day 24.

MUSOCAL MAST CELLS AND SERUM RMCPII CONCENTRATION

Mucosal mast cells showed no significant increase until day 22 (Fig. 4), but there was a progressive loss of granule staining during this time, with fewer and smaller granules being present. Serum RMCPII increased approximately five-fold from days 12 to 22, indicating that the appearance of the granules was caused by sustained degranulation. From day 22 to 24, MMC increased three-fold (p<0-0001), and granules increased in number and intensity of staining. The was associated with a three-fold fall in serum RMCPII to adult values, indicating a decrease in MMC activation.

DISACCHARIDASE AND ALKALINE PHOSPHATASE ASSAYS

Results of disaccharidase and alkaline phosphatase assays are given in Table 2. Lactase activity was maintained during the suckling period from day 12 to 20, but decreased as spontaneous weaning occurred. Sucrase and maltase showed low activity before day 18, and increased before stabilising after day 24.

![Fig. 4](image-url)  Mucosal mast cell count in the small intestine and serum RMCPII concentration in DA rats during weaning from 12 days of age. Each age interval has the mean±SD of six rats.

**Table 2**  Disaccharidase and alkaline phosphatase activities of the small intestine in DA rats during weaning from 12 days of age

<table>
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<tr>
<th>Age</th>
<th>Lactase*</th>
<th>Sucrase*</th>
<th>Maltase*</th>
<th>Alkaline phosphatase+</th>
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</tr>
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<tr>
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</table>

*One unit=μmol of disaccharide hydrolysed/minute/g wet weight; †One unit=μmol of nitrophenol phosphate hydrolysed/minute/g wet weight. Each age interval has the mean (SD) of four to six animals; †Not available.
Alkaline phosphatase increased to a broad peak centred over the mid-weaning period (days 20–26).

Discussion

During weaning, we have described an increase in MLN weight and in jejunal counts of MMC, IEL, eosinophils and goblet cells, and systemic release of RMCPII. These changes were associated with morphological development and crypt cell proliferation and stabilisation. Intestinal maturation was confirmed by changes in disaccharidases and some increase in alkaline phosphatase activity.

Mesenteric lymph node and spleen weights were used as indicators of enteric or systemic immune activity on the principle that a draining lymphoid organ reflects immune activation in the region. In unpublished work, we have recently shown that feeding protein antigen to mice causes increase in MLN weight and reciprocal decrease in spleen weight, and that this is associated with a delayed type hypersensitivity reaction in the MLN as measured by an indirect footpad test. Thus the change in MLN weight in this study provides evidence of increased enteric immune activity during weaning.

We believe that systemic release of RMCPII from MMC indicated mucosal T cell activity. Evidence for this is that a raised serum RMCPII is a sensitive indicator of mucosal graft versus host reaction,9 17 18 which is the exemplary model of a T cell mediated reaction. In addition, the T cell suppressive agent, cyclosporin A, causes a 90% fall in serum RMCPII concentration in normal adult rats,19 implying basal immune stimulation of MMC under physiological conditions. Although MMC are also stimulated by IgE during anaphylaxis,10 11 this is associated with an increase in intestinal permeability,16 rather than a decrease in permeability which occurs during weaning.16 Apart from moderate activity of substance P, MMC are distinguished from connective tissue mast cells by being remarkably resistant to a wide range of secretagogues, such as polyamines and neuropeptides.20 While we cannot altogether exclude a stimulatory effect of substance P and other unknown factors, this is unlikely as we have recently shown that cyclosporin A delays intestinal maturation (unpublished observation), again suggesting that a T cell dependent mechanism may be involved. Moreover, any other mechanism would fail to explain stimulation followed by suppression of RMCPII secretion. Serum RMCPII originates from intestinal MMC, because MMC are preferentially distributed to the gut.21 This systemic release of RMCPII during weaning confirms a previous study.22

All measures of immune activity showed a biphasic response. Thus, MLN weight increased seven-fold up to day 20 and subsequently fell slightly; jejunal cell counts of eosinophils and goblet cells increased until day 24, before stabilising or falling; MMC were activated up to day 22, and this was followed by relative suppression; and IEL were initially low and later showed a delayed rise at day 24. These various measures support the notion that weaning was associated with sequential activation and later suppression of the mucosal immune system. A possible mediator of this suppression of mucosal immune activation could be IEL. Perhaps this is the functional role for IEL during delayed type hypersensitivity reactions in the gut, where their numbers are increased and it could be consistent with their OX8/CD8 suppressor phenotype.23 Some caution is necessary, however, because the nature and in vivo function of IEL is unknown.24 An alternative suppressor system is activated macrophages.25

Mucosal mast cell count and serum RMCPII measurement enabled us to identify activation of MMC in the period from day 16 to 22, even though the MMC count apparently remained stationary. The exponential rise in serum RMCPII showed that this low count was caused by intense degranulation and not low activity. This was also evident in the progressive loss of granule staining in those MMC that remained visible on staining during this period. Thus, although MMC are expanded in immunologically mediated reactions in the gut, a caveat must be added that the apparent number of MMC may fall under intense stimulation. This is also seen in severe graft versus host reaction in rats and contrasts with milder reaction in which MMC increase and serum RMCPII shows a small rise.9

Measurement and expression of the denominator in cell counts of the intestines remain controversial. This reflects the difficulty in defining an invariable reference, whether it be expressed per 100 epithelial cells, per villus/crypt unit, per mm² of mucosa, per mm or area of muscularis mucosae. Marsh has advised expressing counts per area of muscularis mucosae but this assumes that the muscularis mucosae remains unaltered. This assumption seems unjustified particularly during weaning or with protein deficiency,17 because it is likely the muscularis mucosae does alter. Thus, the use of this denominator would seem to have no advantage in these situations. We have chosen, therefore, to rely on differences in cell counts being far greater than any change in muscularis mucosae, and to express cell counts per mm of muscularis mucosae, which can be easily measured using a microscopic graticule.

It is interesting that morphological change and increase in CCPR was synchronous with some changes in mucosal immune activity, such as increase in MLN weight and systemic release of RMCPII from
MMC. In particular, CCPR and serum RMCPII showed an exponential rise from days 12 to 22, before both stabilised. As heightened T cell activity in neonatal graft versus host reaction is known to induce precocious intestinal maturation (before evolving into enteropathy), this would indicate that crypt proliferation may be controlled by T cell activity. This is also supported by the observation that T cell deficient (nude) mice have smaller intestinal crypts and lower CCPR than conventional mice, again suggesting that T cell activity affects crypt proliferation. Our results would extend this concept to the physiological process of weaning maturation. The mucosal immune system may have been activated by bacterial flora that developed before weaning. In addition, there may be some contribution to mucosal stimulation from food antigens either actively ingested or passively absorbed in the dam’s milk. As Ferguson has shown that maturation is partly genetically preprogrammed, we would envisage that this physiological immune reaction would advance this preprogrammed rate of intestinal development.

Although it is known that preweaned animals have Gram positive bacteria, and later Gram negative bacteria appear during or after weaning, our results showed the timing of this change in relationship to immune activity and intestinal maturation. Gram negative bacteria were not identified either on Gram stain of mucosal smears or by culture of gut washing before day 18. It is possible that colonisation with Gram negative bacteria was responsible for activating the suppressive immune phase, leading in turn to stabilisation of maturation. This is suggested because bacterial colonisation of germ free animals generates suppressor cells.

After day 24, the CCPR fluctuated with positive and negative swings as it stabilised suggesting that a negative feedback system was operating, although it is not possible to completely exclude an effect of random variation. The presence of such a negative feedback system would, however, still be necessary to explain stabilisation of exponential rise in crypt cell proliferation after day 24. The presence or absence of fluctuations is not crucial to demonstration of a negative feedback system, as these would be dependent on the degree of dampening of the system. A negative feedback system has already been shown in the intestine during the recovery phase of adult mice treated with cytarabine by Wright and Al-Nafussi. Although the previous authors have also made a careful study of cell kinetics during weaning in the mouse, their study was terminated before maximum CCPR was achieved (at 70% of adult values), and hence fluctuations (if present) were not observed.

Taken together, our results suggest that maturation of the small intestine during weaning is controlled in part by activation of the mucosal immune system. The latter may be because of the development of bacterial flora or to the presence of food antigens, and it may be followed by a period of immune suppression leading to a final stabilisation of crypt cell production to adult levels.

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