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An investigation into the reversibility of the morphological and cytokinetic changes seen in the small intestine of riboflavin deficient rats

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Abstract

***Background*—Impaired iron handling in riboflavin deficiency is thought to be partially a result of significant morphological and cytokinetic changes within the small intestine.**

***Aims*—The aim of the study was to find out if the responses of the rat small intestine to riboflavin deficiency induced at weaning could be reversed upon repletion.**

***Subjects*—48 female weanling Wistar rats were used for the purpose of the study.**

***Methods*—Rats were fed a riboflavin deficient diet or a complete control diet for a period of five weeks followed by a repletion period of up to three weeks. Rats were killed on day 0, 2, 7, or 21 of repletion. The duodenum was removed and fixed for subsequent analysis.**

***Results*—Five weeks of riboflavin deficiency significantly changed the morphology and cytokinetics of the duodenum; the changes were not reversed within the 21 day repletion period despite biochemical evidence for a correction of the deficiency.**

***Conclusions*—The results show that the small intestine cannot readily recover from a period of riboflavin deficiency induced at weaning, supporting the notion that the weaning period is a critical time for gastrointestinal development and highlighting the importance of adequate nutrition during infancy.**

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Keywords: riboflavin depletion and repletion, intestinal morphology, rats.

Human and animal studies have shown riboflavin deficiency to have significant effects on iron absorption and loss,[1–3] which may be partially explained by the effects of riboflavin deficiency on the structure and cytokinetics of the small intestine.[4–6] The morphology of the small intestine alters in response to riboflavin deficiency induced from weaning; crypt depth increases and villi increase in length and width.[4,5] The increase in villus length can be accounted for by a proportional increase in the number of cells along a single villus column.[5] The cytokinetic response of the small intestine to riboflavin deficiency induced at weaning includes increased crypt cell production rate[4] and increased distance travelled by the leading edge of newly replicated cells along the villus.[6] A further effect of riboflavin deficiency induced at weaning is an inhibition of an increase in villus number in the duodenum, observed within three weeks post-weaning in rats fed a complete diet.[6] Crypt and villus hypertrophy may be an adaptive response to a reduced surface area resulting from fewer villi.

The immediate weaning period is a time of extreme change in terms of gastrointestinal growth rate. The effects of a five week period of riboflavin depletion induced at weaning followed by a three week repletion period were investigated to assess the longterm impact of riboflavin deficiency during weaning and to determine how readily the morphological and cytokinetic changes could be reversed.

Methods

Animals

Forty eight female weanling Wistar rats (Sheffield strain), weighing 45–60 g were used for the study. The rats were weighed at the start of the experiment, paired by weight, and then allocated to one of two dietary groups: riboflavin deficient or control. Rats assigned to the riboflavin deficient group were fed a semi-synthetic diet containing (g/kg): arachis oil 30, sucrose 700, casein 200 (providing 0·52 mg riboflavin/kg diet), a riboflavin free vitamin mixture 0·23, and Briggs salt mixture 50, (Seaford Laboratories, Seaford, East Sussex).[7] Control rats were fed the same diet but supplemented with 15 mg riboflavin/kg diet. The rats were weighed and fed daily at 0930. The riboflavin deficient animals were allowed to feed ad libitum between 0930 and 1630. Feeding rats a riboflavin deficient diet from weaning leads to inanition and is also associated with a reduction in the efficiency of food energy utilisation.[6,8] As the development of the gastrointestinal tract is body weight dependent,[9] the control rats were weight matched to their riboflavin deficient partners by restricting the food available to the control group. Food was available to the control rats between 0930 and 1630. The rats were individually housed in wire bottomed cages to minimise coprophagy and permitted free access to tap water. Standard laboratory conditions were maintained throughout: a 12 hour light-dark cycle, a mean temperature of 21°C, and a mean humidity of 45%.

Blood was collected at the start of the experiment and at 14 day intervals throughout the deficiency period. All blood samples were collected between 0930 and 1230 after a standard overnight fast. Rats were anaesthetised with an intraperitoneal injection of Hypnorm

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(Janssen Pharmaceuticals, Oxford) (0·2 ml/kg body weight). The lower limb was shaved to expose the saphenous vein, which was punctured with a scalpel blade. One hundred μl of blood were collected from each animal into a heparinised tube, which was kept on ice prior to processing. The rats were allowed to recover and time of food availability extended to permit adequate food consumption. Blood samples were centrifuged and the plasma discarded, the erythrocytes were washed three times in an equal volume of saline (9 g NaCl/l), and stored at −20°C in three volumes of distilled water. The haemolysate was subsequently used for the analysis of riboflavin status expressed in terms of the activation coefficient for the flavin adenine dinucleotide (FAD) dependent enzyme erythrocyte glutathione reductase (EC 1.6.4.2) (EGRAC) measured using a spectrophotometric assay modified for use on the Cobas Bio Autoanalyser (Roche Diagnostics, Welwyn Garden City).[10]

The rats were maintained on their diets for 35 days at which time six weight matched pairs of rats were given an intraperitoneal injection of bromodeoxyuridine (50 mg/kg body weight prepared in 500 μl distilled water) (Sigma Chemical, Poole, Dorset). Twenty four hours later, after the standard overnight fast the rats were anaesthetised with chloroform and exsanguinated by cardiac puncture. Blood was collected from the cardiac puncture into heparinised tubes, the liver was removed, washed in saline, blotted dry, and frozen at −20°C. The liver was later used for the measurement of liver flavins.[11] The small intestine was dissected out of each animal, the length measured, and two 20 mm lengths of duodenum were cut 40 mm distal to the pylorus. The sections were opened along the mesentery, pinned out onto dental wax, and fixed for 24 hours in 40 g/l para-formaldehyde prepared in 0·01 mol/l phosphate buffered saline pH 7·4 (PBS). The first section was processed for paraffin wax embedding and 5 μm thick longitudinal sections cut. The second section was transferred to ethanol (700 ml/l) and used for villi analysis.

The remaining 18 riboflavin deficient rats were gavaged with flavin mononucleotide (10 mg/kg body weight prepared in 200 μl distilled water) to reverse the riboflavin deficiency state. The riboflavin deficient group were fed the control diet ad libitum during the seven hour feeding period and the control group continued to be weight matched. Six randomly selected weight matched pairs of rats were killed on days 2, 7, or 21 after repletion having been given an intraperitoneal injection of bromodeoxyuridine 24 hours earlier.

Calcium of villus number

A 10 mm length segment of duodenum was stained using the Feulgen staining reaction.[12] The segment of tissue was placed on a microscope slide with a drop of acetic acid (450 ml/l), secured with a cover slip, and viewed under a light microscope. The arrangement of villi in parallel rows allowed the number of villi within the 10 mm length to be calculated by multiplying the number of villi per row by the number of villi rows. Five separate counts were made on the same piece of duodenum.

Measurement of cell migration and crypt and villi morphology

Bromodeoxyuridine is a thymidine analogue, which is incorporated into DNA during the synthetic phase (S-phase) of the cell cycle; monoclonal antibodies to bromodeoxyuridine[13] can be used in conjunction with DNA denaturation and immunoperoxidation to detect the presence and movement of newly replicated cells. Bromodeoxyuridine labelled cells were detected in histological tissue sections using a modification of the method described by Wynford-Thomas and Williams.[14] Tissue sections were immersed in 3 ml/l H_2O_2 prepared in 500 ml/l methanol to block endogenous peroxidase activity, and washed in PBS. Each section was covered with 50 μl of 0·4 g/l solution of pepsin prepared in 0·1 mol/l HCl, for 25 minutes and incubated in 2 mol/l HCl for 30 minutes at 37°C to denature the DNA. Sections were neutralised with 0·1 mol/l di-sodium tetraborate and after two, five minute washes in PBS were treated with 150 μl of secondary biotinylated antimouse antibody conjugated to horseradish peroxidase at a dilution of 1 in 100 in horse serum. After two, five minute washes in PBS the sections were incubated with Avidin Biotin Complex (Vector Laboratories, Peterborough, UK) for 30 minutes and after two further five minute washes with PBS the sites of antibody attachment were identified by a 15 minute incubation with peroxidase substrate (amino-ethyl carbozole) kit (Vector Laboratories, Peterborough, UK). The sections were washed for five minutes in water before mounting with glycerol. The distance travelled by bromodeoxyuridine labelled cells from the crypt: villus junction along the villi was measured using Image Analysis (Seescan Analytical Services, Cambridge, UK) and used as an index of the enterocyte migration rate. Villi lengths and crypt depths were also measured on the same histological sections. Five measurements were made for each parameter.

Statistical analysis

Two way analysis of variance was used to investigate the effects of diet and time on all variables throughout the experiment. Where differences were identified a Mann-Whitney U test was performed.

Results

Body weight and dietary data

Table I shows data for final mean body weight, mean daily food consumption, and weight gain/g diet. There was no significant effect of diet on the final mean body weights of the rats ($p=0·975$) but an effect of time was identified

TABLE I *Effect of diet on final body weight (g), daily food consumption (g) and weight gain (g)/g diet*†

Variable	Group	Days from repletion: 0 (n=6)	2 (n=6)	7 (n=6)	21 (n=6)
Final body weight	RD	144 (5·2)	124 (9·3)	139 (4·9)	154 (2·1)
	WM	144 (4·8)	126 (8·0)	137 (5·0)	153 (2·4)
Daily food consumption	RD	11·7 (0·13)	11·5 (0·24)	11·7 (0·08)**	11·4 (0·26)**
	WM	11·2 (0·49)	10·3 (0·62)	10·1 (0·19)	9·7 (0·29)
Weight gain (g)/g diet	RD	0·19 (0·007)	0·17 (0·009)	0·17 (0·008)	0·17 (0·008)*
	WM	0·20 (0·007)	0·19 (0·003)	0·19 (0·007)	0·21 (0·009)

†For details of diets and procedures see text. Significantly different from weight matched control animals (Mann-Whitney U test) *p<0·05, **p<0·01.
RD=rifloflavin deficient, WM=weight matched. Data shown as mean (SEM).

TABLE II *Effect of diet on EGRAC values and liver FAD concentrations (μg/g wet weight)*†

Variable	Group	Days from repletion: 0 (n=6)	2 (n=6)	7 (n=6)	21 (n=6)
EGRAC	RD	2·12 (0·05)**	1·51 (0·06)	1·44 (0·03)	1·39 (0·05)
	WM	1·32 (0·06)	1·32 (0·07)	1·37 (0·01)	1·30 (0·02)
FAD	RD	8·85 (0·62)**	11·85 (0·90)**	17·97 (0·53)	17·88 (1·39)
	WM	18·77 (0·17)	20·07 (0·58)	21·60 (0·58)	18·41 (0·71)

†For details of diets and procedures see text. Significantly different from weight matched control animals (Mann-Whitney U test) *p<0·05, **p<0·01.
Data shown as mean (SEM).

TABLE III *Effect of riboflavin deficiency followed by repletion on the crypt and villi morphology, villus number within a 10 mm length of duodenum and on enterocyte migration*†

Variable	Group	Days from repletion: 0 (n≥5)	2 (n≥5)	7 (n≥5)	21 (n≥5)
Villus length	RD	578 (21·4)*	611 (16·7)*	650 (50·7)	619 (38·4)*
	WM	482 (33·1)	547 (21·7)	550 (23·9)	525 (18·6)
Crypt depth	RD	188 (5·3)*	189 (9·4)	192 (2·9)*	198 (8·9)*
	WM	168 (5·4)	173 (8·7)	173 (4·5)	167 (3·9)
Villus number	RD	1322 (46·4)**	1236 (27·0)**	1373 (18·1)**	1327 (63·0)*
	WM	1662 (41·0)	1673 (54·0)	1677 (69·5)	1608 (48·1)
Leading edge (μm)‡	RD	197 (26·7)*	185 (26·7)**	168 (13·3)*	173 (17·2)*
	WM	117 (14·4)	95 (7·9)	134 (7·6)	104 (15·0)

†For details of diets and procedures see text. Significantly different from weight matched control animals (Mann-Whitney U test) *p<0·05, **p<0·01. ‡Distance of the leading edge (that is, cohort of cells furthest from the crypt:villus junction) from the base of the villus, 24 hours after bromodeoxyuridine. Data shown as mean (SEM).

(p<0·001) (two way ANOVA). The Mann-Whitney U test showed the final mean body weight to be significantly greater after 21 days repletion than after two and seven days repletion for both the groups (p<0·05). The final mean body weights of rats killed after a five week period of riboflavin depletion (that is, at repletion day 0) were greater than rats killed after two and 14 days of repletion. This is not reflective of weight loss during repletion but simply that those rats randomly selected to be killed on repletion day 0 were those that had been the heaviest at the beginning of the experiment. A significant effect of diet on mean daily food consumption (g) and mean weight gain per gram of diet consumed (p<0·001, p<0·001) was identified but not of time (p=0·070, p=0·203) (two way ANOVA). The riboflavin deficient group of rats who were repleted for seven and 21 days had significantly higher mean daily food consumption than their weight matched control partners (p<0·01). Weight gain per gram of diet was significantly lower only in the riboflavin deficient group repleted for 21 days (p<0·05) (Mann-Whitney U).

Riboflavin status of the rats

There was a significant effect of diet and time on the EGRAC values measured in a peripheral blood sample of the two groups over the eight week experimental period and a significant interaction between these two variables (p<0·001) (two way ANOVA). The rats assigned to the riboflavin deficient and weight matched control groups had mean (SEM) EGRAC values of 1·34 (0·02) and 1·31 (0·05) respectively at the start of the experiment. These values were normal and were not significantly different (Mann-Whitney U). The EGRAC values of the riboflavin deficient rats increased significantly from the start of the experiment and were significantly higher than the control rats by day 14. All values in the riboflavin deficient group were greater than 1·4, which is indicative of a biochemical deficiency of riboflavin. A significant difference between the two dietary groups was maintained throughout the duration of the 35 day deficiency period.

Table II gives the final mean EGRAC values and mean liver FAD concentrations for each group of animals at the time of death.

Repletion resulted in a significant fall in EGRAC values in the riboflavin deficient rats, which were not significantly different from control values by repletion day 2. Two way analysis of variance highlighted a significant effect of diet ($p<0.001$) and time ($p<0.001$) on liver FAD concentrations and a significant interaction between these two variables ($p<0.001$). Liver FAD concentrations were significantly lower in the riboflavin deficient group than in the control group on day 0 and remained so on day 2 of repletion. Concentrations rose upon further repletion and reached control values by day 7. Liver FAD concentrations in the control group did not change over time, in contrast with a significant increase in the repleted group by day 7, compared with the day 0 value.

Morphology and cytokinetics of the intestine

Table III shows the results of the morphological and cytokinetic analysis. There was a significant effect of diet on villus number, villus length, crypt depth, and distance travelled by the leading edge along the villus ($p<0.001$ for all variables); there was no significant effect of time ($p=0.498$, $p=0.114$, $p=0.897$, and $p=0.844$ respectively) and no significant interaction between diet and time ($p=0.405$, $p=0.916$, $p=0.663$, and $p=0.404$ respectively). The Mann-Whitney U test found the number of villi within the experimental group to be significantly lower than the control group after five weeks on a riboflavin deficient diet, which remained so throughout the period of repletion. Similarly, the distance travelled by bromodeoxyuridine labelled enterocytes along the villi was significantly greater in the riboflavin deficient group than in the control group at all points of measurement. The distance travelled by the leading cohort of cells along the villus fell slightly over the period of repletion, from mean (SEM) 197 (26·7) μm to 173 (17·2) μm, but remained significantly higher than control values. Villus height and crypt depth were significantly greater in the riboflavin deficient group on all days of measurement except repletion day 7 in the villus and repletion day 2 in the crypt.

Discussion

This study shows that riboflavin deficiency induced at weaning in the Wistar rat arrests normal gastrointestinal development. The morphological and cytokinetic effects of riboflavin deficiency could not be reversed within a three week repletion period despite a complete biochemical reversal of the deficiency state. The results highlight the importance of dietary riboflavin during the weaning period and support the concept of a fixed villus number after weaning.

Riboflavin deficient rats gavaged with FMN and fed a complete diet were rapidly repleted with riboflavin, shown by EGRAC values and liver FAD concentrations being normalised within 48 hours and seven days of the initial repletion respectively. Tillotson and Sauberlich[15] studied the effects of removing riboflavin from the diet followed by feeding a diet containing graded amounts of riboflavin, on the activation coefficient of erythrocyte glutathione reductase and concluded that this was a particularly sensitive indicator of riboflavin intake. EGRAC values of riboflavin deficient rats fell within five days of feeding diets of varying riboflavin concentrations, the magnitude of the fall reflecting the concentration of riboflavin in the diet. The response of tissue FAD concentrations to riboflavin depletion and repletion is gradual and less prone to daily fluctuations. EGRAC values, while offering a useful functional assay for riboflavin status are less valuable as an indicator of tissue levels, which are best measured directly and are more a measure of longer term status. In this experiment, in addition to supplying 15 mg of riboflavin per kg of diet the rats were given a single intragastric dose of FMN on the first day of repletion to correct any deficiency of the rats as rapidly as possible. Both EGRAC values and liver FAD concentrations provide convincing evidence of complete repletion within seven days.

Villus number is a particularly stable component of the gastrointestinal tract. Clarke[16] reported that the total villus number within the entire small intestine did not change significantly during early life. Forrester[17] drew similar conclusions from measurements made in the small intestine but excluding the duodenum. In contrast, we have reported a significant increase in villus number within a 10 mm length of duodenum in control rats within the first three weeks after weaning.[6] The discrepancy between these reports could be explained if the increase in villus number is confined to the duodenum. Riboflavin deficiency inhibits this developmental increase in villus number in the duodenum and the data from this study support this.

There has been very little work on the mechanism of villus formation in the small intestine. Columbre and Columbre[18] and Burgess[19] have investigated villus development in the chicken small intestine and concluded that villi arise from previllus ridges. Dunn[20] described the prenatal development of villi in the rat small intestine but a postnatal increase in villus number has not been investigated. The mechanism by which the duodenum increases the number of villi during the first three weeks after weaning in control rats, and by which riboflavin deficiency halts this is unclear.

Differences in villi development in the rat small intestine as a consequence of dietary manipulation at weaning have previously been reported in 10 mm^2 jejunum and ileum by Tasman-Jones *et al.*[21] Rats fed from 12 weeks from weaning on a diet containing no dietary fibre had more villi per unit area and all villi remained morphologically underdeveloped. There was a reduction in the number of villi per 10 mm^2 of intestine with age, due to the counts being made per unit area; as the dimensions of the villi increase they occupy more space thereby reducing the number per unit

area. In contrast, in this experiment the number of villi were not counted in a defined area; the number of rows of villi and the number of villi per row were counted in a 10 mm length of duodenum. These counts were used to calculate the total number of villi per unit length of duodenum. The dimensions of the crypts and villi increase postnatally and are accommodated by increased intestinal circumference. The method described permits a comparison of the number of villi per unit duodenal length in the two dietary groups and does not take into account any difference there might be in circumference. There was an increase in the number of villi per 10 mm length of duodenum in normal animals, which was inhibited by feeding a riboflavin deficient diet at weaning. The villi formed in the riboflavin deficient animals have increased dimensions.[5] The increasing circumference of the intestine during development would accommodate the increased number of villi in the control rats and permit the expansion of villi dimensions in the riboflavin deficient rats.

Correcting the riboflavin status to control values did not restore villus number to control values, supporting the hypothesis that villus number once fixed in early adulthood cannot readily be changed.[16 17] A permanent reduction of villus number associated with a reduced surface area would have adverse effects on the absorptive capacity of the duodenum and may have detrimental effects on the longterm nutritional status of a person. The associated increase in crypt and villi dimensions may be an adaptive response to combat impaired villus number. There are examples in the literature to support the suggestion that villus dimensions may change as an adaptatory response, whereas villus number does not.[16 17 22] The response of the small intestine to resection provides the classic example of the adaptatory response to a reduced number of villi. Crypt and villus dimensions within the residual portion of the intestine increase whereas villus number remains constant.[17 23]

Confirming our previous findings[5] the villus length and crypt depth were significantly greater in rats fed a riboflavin deficient diet for five weeks from weaning compared with age and weight matched control animals. This study shows that villus height and crypt depth remained increased in the riboflavin deficient animals throughout the repletion period with the exception of the villus height on repletion day 7 and crypt depth on repletion day 2 when the values just failed to reach significance. These data show that the effects of riboflavin deficiency on the morphology of the small intestine cannot be reversed within a three week repletion period, suggesting that abnormal morphology persists despite tissue repletion. Similarly the distance travelled by the leading edge of newly replicated enterocytes was greater in the riboflavin deficient group than the control animals and remained significantly higher even after feeding a complete diet for three weeks. A significant effect of the length of time of repletion on the migration of enterocytes was not detected although there did seem to be a gradual reduction in the distance travelled by the enterocytes during the three week repletion period of the riboflavin deficient rats. This gradual decline in the distance travelled by enterocytes may indicate that over a longer repletion period or in animals in which the deficiency state was less severe we might see a reversal of these cytokinetic changes induced by riboflavin depletion.

Because of the three dimensional nature of the villus, villus height does not necessarily reflect surface area particularly in abnormally shaped villi.[24] However, we reported an association between villus height, villus number, and total mucosal DNA.[6] The events associated with riboflavin deficiency induced at weaning imply that an initial reduction in surface area is progressively compensated for by increased crypt and villus dimensions. In the immediate period after weaning onto a riboflavin deficient diet rats fail to increase the number of villi within the duodenum and this is associated with a significantly lower total mucosal DNA than the control rats but an unchanged villus length. By three weeks of age, despite no change in villus number, total mucosal DNA reaches control values and the villi have lengthened. Equality of mucosal DNA indicates equality of total cellularity but not necessarily of enterocytes. The failure to increase the number of duodenal villi and the transient failure to increase the total mucosal cellularity of the small intestine of the riboflavin deficient rats would predictably be associated with a reduced absorptive surface area and hence an impaired absorptive capacity. Although the mucosal cellularity is restored to control values upon prolonged riboflavin deficiency this seems to be achieved by the development of abnormally shaped villi that have an increased enterocyte migration. Such events may be associated with an altered rate of enterocyte differentiation and a reduction in the proportion of mature enterocytes populating the villi. Therefore despite an element of structural adaptation to riboflavin deficiency, the functional absorptive capacity of the intestine may remain compromised. As the effects of riboflavin deficiency induced at weaning are not readily reversed upon repletion the functional capacity of the small intestine may never fully recover from a period of riboflavin deficiency at weaning.

Intestinal morphological changes are at their greatest in the young rat (30–60 g), during the period that follows weaning[25]; there is a pronounced increase in the crypt and villus epithelial cell populations,[26] crypt cell production rate, villus length, crypt depth, and crypt to villus ratio.[27] These changes seem to be partially related to changing diet because proliferative changes within the small intestine at this age can be delayed by delaying the onset of free feeding.[28] Our results support the evidence that the nature of the diet after weaning is important in terms of intestinal development and dietary change during this period can have irreversible consequences.

This study highlights the weaning period as a critical period for normal intestinal development. Investigation into the mechanism by which villi increase in number at weaning would provide further insight into normal intestinal development.

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