# Upper Intestinal Mucosal Proliferation in the Newborn Guinea Pig: Effect of Composition of Milk Feeds

L. T. WEAVER AND A. LUCAS

University of Cambridge & Medical Research Council Dunn Nutritional Laboratory, Milton Road, Cambridge, England

**ABSTRACT.** To examine the effects of first enteral feeds on the development of the gastrointestinal tract, the changes in upper intestinal mucosal morphology and kinetics were studied during the 1st wk of postnatal life in neonatal guinea pigs. Animals were reared either on mother's milk or on a cow's milk formula isocaloric with guinea pig milk. Mucosal crypt-villus architecture was measured by microdissection, and mucosal kinetics were measured using a metaphase arrest technique. Comparable growth was achieved between the two feeding groups. There were no significant differences in the villus heights, crypt depths, or crypt:villus ratios between the naturally fed and formula-fed guinea pigs. The formula fed had a crypt cell production rate twice as high as the naturally fed animals throughout the study period ($p < 0.001$). The higher mucosal proliferation rate of the formula-fed animals may be due to the absence of growth-modulating factors in this milk, or a regenerative response to "damage" of the upper intestinal mucosa by cow's milk proteins. (*Pediatr Res* 22: 675–678, 1987)

### Abbreviations

**CMF**, cow's milk formula
**CCPR**, crypt cell production rate
**EGF**, epidermal growth factor

We have previously described marked differences in the passive intestinal permeability of breast and CMF-fed human neonates and newborn guinea pigs (1). The CMF-fed guinea pigs showed a persistently higher intestinal permeability to lactulose than the breast-fed guinea pigs throughout the 1st wk of life. These changes were not associated with differences in upper intestinal mucosal histology between the two feeding groups.

Such differences in passive intestinal permeability in response to composition of enteral feeds may be due to differences in rate of mucosal turnover, ultrastructural morphology, or enterocyte differentiation and function (2).

In the absence of differences in mucosal morphology in the newborn guinea pig we chose to study the proliferative response of the upper intestinal epithelium to milk feeding to determine whether the marked differences in intestinal permeability of the naturally and CMF-fed animals were associated with differences in enterocyte turnover. Increased CCPR may lead to a greater

villus population of immature enterocytes forming an epithelium with altered permeability characteristics.

We used a metaphase arrest technique to measure upper intestinal mucosal proliferation during the 1st wk of postnatal life. As in our earlier study we chose the guinea pig as a model with which to investigate the differences in passive intestinal permeability seen between breast- and CMF-fed human infants (1), because, like man, it acquires the majority of its circulating antibody transplacentally and undergoes intestinal closure soon after birth (3). Moreover it is comparatively mature and independent at birth and may be separated from its mother and reared independently on an artificial milk formula.

## ANIMALS AND METHODS

One hundred sixteen newborn guinea pigs were studied during the 1st wk of life. All were born at term of locally bred Dunkin Hartley guinea pigs which were reared in pens of 12, receiving a diet of SG1V pellets (Dixons, Ware, England), cabbage, and water *ad libitum*. After birth naturally fed litters were caged with their dam and CMF-fed litters with a surrogate nonlactating female.

*Nutrition.* Newborn guinea pigs were divided into two feeding groups. Sixty-five naturally fed animals remained with their dam from birth and were allowed to suckle *ad libitum*. In common with their mothers they were allowed access to solid pellets.

Fifty-one CMF-fed animals were separated from their dam within 15 h of birth and fed with a cow's milk-based formula. The composition of 136 samples of expressed guinea pig milk obtained from lactating dams 2 to 13 days postpartum was analyzed. A formula composed of pasteurised cow's milk and Casilan milk protein (Farley's Ltd, Plymouth, England) was designed to mimic the composition of natural guinea pig milk during mid lactation (Weaver LT, Landymore-Lim AFN, Hudson GJ, unpublished data) (Table 1). During the first few days of life they received feeds by syringe gavage five to eight times a day to achieve a daily milk intake of 20 to 30 ml. Thereafter they were allowed free access to bowls containing the milk formula, as well as solid pellets.

*General design of experiments.* Newborn guinea pigs were reared as above until they were killed at intervals up to 8 days of postnatal life. Day of birth was called day 1. On the day of the experiment the young were given 3 μg demecolcine (Colcemid, Gibco, Paisley, Scotland) by intraperitoneal injection at 1400 h. Preliminary studies showed that this dose arrested mitoses at metaphase without anaphase escape or degeneration, and with linearity of metaphase accumulation (5). Animals were killed at 30-min intervals thereafter by cervical dislocation. The abdomen was opened by longitudinal midline incision and the gut was removed intact from cardia to anus. One-cm length specimens were excised 5 cm distal to the pylorus, opened longitudinally

and laid, mucosal side up, on a card before preservation in Clarke's fixative (75% ethanol, 25% acetic acid).

*Microdissection.* After fixation in Clarke's solution for 24-h specimens were preserved in 75% ethanol until staining with Schiff reagent by the Feulgen reaction (5). This was preceded by rehydration through descending concentrations of ethanol and hydrolysis with M HCl at 60° C for 6 min. Individual crypt-villus units were dissected under a stereomicroscope at ×20 magnification, suspended on a slide under a cover slip in 45% acetic acid, and the following measurements were made.

*Crypt-Villus Architecture.* The heights of 10 well-orientated villi and the depths of 10 mature crypts per animal were measured under ×40 and ×250 magnification respectively using a 1-mm graduated eyepiece graticule. The ratio of the number of crypts to each villus was measured as described by Wright and Irwin (6).

*Crypt Cell Production Rate.* Gentle pressure was applied to the cover slip to produce squash separation of individual crypts. The number of well stained arrested metaphase figures per 10 complete undamaged crypts was measured. A litter of at least three animals was used per measurement. The CCPR was calculated from the slope of the plot of the mean number of metaphase arrests per crypt per animal against time after administration of demecolcine (6, 7). Only results in which a correlation coefficient of >0.8 was obtained were used.

*Statistics.* The results of measurements of crypt-villus architecture were expressed as mean villus heights, crypt depths, and crypt:villus ratios of animals of each age and feeding group calculated from 10 measurements per animal. The unpaired Student's $t$ test was used to test the significance of differences in mucosal achitecture between the two feeding groups.

The significance of the differences between feeding groups in mucosal proliferation rates with time was tested by deriving equations to describe the relation between CCPR and postnatal age, and then determining whether there were significant differences between the slopes and intercepts.

## RESULTS

*Growth.* Comparable growth was achieved between the two feeding groups (Table 2). The number of animals studied on each day is shown. All animals thrived and none showed any adverse effects of CMF feeds.

*Crypt:villus architecture.* There were no significant differences in the villus heights, crypt depths, or crypt:villus ratios between the naturally and CMF-fed animals and there were no changes with increasing postnatal age (Table 3).

*Crypt cell production rates.* Ten naturally fed and 12 CMF-fed litters were studied. Figure 1 shows the CCPRs of the naturally and CMF-fed animals plotted against postnatal age.

The slope of the regression line for the naturally fed guinea pigs was 0.11 and that for the CMF-fed animals 0.16. There was no significant difference between these two slopes. However, there was a significant difference between the intercepts ($p <$ 0.001): that of the naturally fed was 1.9 and that of the CMF-fed was 3.7. These results indicate that the CMF-fed animals had a CCPR almost twice that of the naturally fed throughout the study
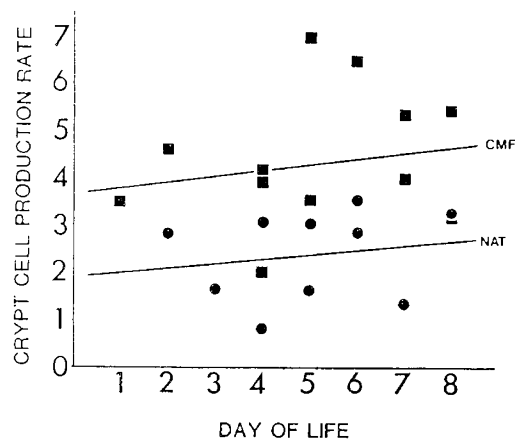
Table 1. *Composition of natural guinea pig milk and CMF*

| Composition | Expressed guinea pig milk | Artificial formula* |
|---|---|---|
| Protein (g/100 ml) | 8.1 | 8.1 |
| Fat (g/100 ml) | 4.0 | 3.8 |
| Carbohydrate (lactose) (g/100 ml) | 4.3 | 4.6 |
| Sodium (mmol/liter) | 18 | 17 |
| Potassium (mmol/liter) | 39 | 23 |
| Energy (kcal/100 ml) | 85 | 84 |
| Osmolality (mosmol/kg) | 285 | 288 |

* Cow's milk plus "Casilan" milk protein.

Table 2. *Body wt (g) of naturally and CMF-fed guinea pigs during first 8 postnatal days of life*

| | Naturally fed | | | CMF-fed | | |
|---|---|---|---|---|---|---|
| Day | n | Mean | SD | n | Mean | SD |
| 1 | 2 | 92 | 4 | 3 | 95 | 5 |
| 2 | 7 | 91 | 22 | 4 | 104 | 3 |
| 3 | 11 | 92 | 17 | 2 | 95 | 7 |
| 4 | 12 | 97 | 16 | 13 | 94 | 18 |
| 5 | 11 | 101 | 14 | 14 | 100 | 19 |
| 6 | 14 | 95 | 20 | 4 | 82 | 11 |
| 7 | 4 | 108 | 17 | 7 | 105 | 15 |
| 8 | 4 | 116 | 18 | 4 | 118 | 7 |



Fig. 1. Crypt cell production rates (arrested metaphases/crypt/h) of naturally fed (*Nat* ●) and CMF-fed (■) guinea pigs during the 1st wk of postnatal life. The difference between the intercepts was significant at $p$ < 0.001.

Table 3. *Villus and crypt morphometry (mean and SD) of naturally and CMF-fed guinea pigs during first 8 days of postnatal life**

| | Naturally fed | | | | CMF fed | | | |
|---|---|---|---|---|---|---|---|---|
| Day | n | VH ($\mu$m) | CD ($\mu$m) | C:V | n | VH ($\mu$m) | CD ($\mu$m) | C:V |
| 1 | 2 | 1014 (90) | 160 (8) | 6.0 (0.3) | 3 | 981 (29) | 163 (7) | 5.6 (1.2) |
| 2 | 7 | 1021 (118) | 131 (8) | 5.2 (0.7) | 4 | 1114 (111) | 174 (12) | 7.1 (0.6) |
| 3 | 11 | 962 (154) | 154 (20) | 5.1 (0.6) | 2 | 1121 (95) | 139 (10) | 5.3 (0.4) |
| 4 | 12 | 1069 (169) | 169 (24) | 4.9 (1.0) | 13 | 815 (178) | 140 (20) | 4.6 (0.4) |
| 5 | 11 | 985 (145) | 155 (29) | 5.0 (0.8) | 14 | 1057 (112) | 163 (28) | 4.6 (0.7) |
| 6 | 14 | 911 (166) | 166 (21) | 5.6 (0.7) | 4 | 976 (83) | 179 (18) | 5.7 (0.5) |
| 7 | 4 | 985 (100) | 193 (21) | 5.4 (0.2) | 7 | 841 (232) | 170 (31) | 4.6 (0.6) |
| 8 | 4 | 934 (63) | 206 (13) | 6.0 (0.4) | 4 | 841 (122) | 157 (63) | 4.4 (0.1) |

* VH, villus height; CD, crypt depth; C:V, crypt:villus ratio.

period. The CCPR of both feeding groups increased gradually during the first 8 days of postnatal life.

## DISCUSSION

Our study shows significant differences in the upper intestinal mucosal proliferative response of newborn guinea pigs to composition of enteral feeds. From the initiation of enteral feeding throughout the 1st postnatal wk the CMF-fed animals showed a persistently higher CCPR than the naturally fed animals. There was a gradual rise in CCPR with increasing age in both feeding groups. Using microdissection for the measurement of crypt-villus architecture we have showed no morphological differences between the two feeding groups, nor any histological changes with increasing postnatal age.

Adaptation of the intestinal mucosa to the initiation of enteral nutrition may be measured as changes in crypt-villus architecture and epithelial cell turnover. The metaphase arrest technique allows a direct measure of the rate of entry of new cells into mitosis, and in a wide range of conditions it has been shown to agree closely with tritiated thymidine labeling techniques (7). Using the microdissection techniques described both villus height and crypt depth, and the number of crypts serving each villus (C:V ratio) were measured. The rate of new cell production per crypt (CCPR measured by metaphase arrest) multiplied by the crypt:villus ratio is a measure of the net villus influx. In the absence of differences in villus height, crypt depth, and C:V ratio between the two feeding groups, or with increasing postnatal age, the elevated CCPR of the CMF-fed animals represents a true increase in mucosal turnover rate in response to this milk formula.

Maturation of the gastrointestinal mucosa occurs under the influence of both intraluminal and extraluminal factors (8). Among the former composition of milk feeds may be the most important.

The milk of mammals contains a range of trophic factors, hormones, and other biologically active substances (9) which may exert growth-modulating effects on the gastrointestinal mucosa (10). Their concentrations are highest in colostrum (9) and it has been observed that epithelial cell lines grow more readily in colostrum than in older milk (11). Recently the presence of endogenous milk factors which modify EGF binding to enterocytes has been proposed to account for the decrease in EGF binding after birth (12). It is therefore possible that our findings of a greater CCPR in the formula-fed animals were due to an absence of modulating factors in the artificial milk, or even the presence of inhibitors in natural milk, which modified the control of mucosal proliferation.

An alternative explanation is that CMF caused an elevated CCPR as a regenerative response to "damage" of the small intestinal mucosa by cow's milk proteins. Such damage is suggested by the elevated passive intestinal permeability described in our earlier study (1) and may be mediated by binding of cow's milk proteins to an immature epithelial surface with disruption of mucosal integrity.

Support for this hypothesis has been presented in a model of the mechanism by which the food lectin, concanavalin A, given by mouth to neonatal guinea pigs, induced a brisk increase in CCPR in association with an elevated passive intestinal permeability to lactulose (13). Binding of concanavalin A to individual microvillar components and accessibility of the lectin to both jejunal crypt and villus cells, with disruption of microvillus architecture, were also shown, demonstrating how an ingested protein may cause such changes as described herein.

Further support for this hypothesis comes from studies of the neonatal rat. Stern *et al.* (14) have shown that both binding and uptake of milk proteins by the intestinal mucosa is greater in suckling than adult animals. Such differences in mucosal protein handling are associated with differences in microvillous membrane composition between immature and mature animals (15).

The intraluminal environment may be altered not only by composition of feeds but also by bacterial flora. Bullen (16) has shown how sensitive the proliferation of *Escherichia coli* is in both the small and large intestines of guinea pigs to composition of feeds. Such change in bacterial populations may alter mucosal architecture, as has been shown by Sprinz (17) in studies of germ-free adult guinea pigs. The part played by bacterial flora in our study is not shown.

Extraluminal factors may also be involved in the findings we have described. The effects of maternal deprivation were minimised by rearing the CMF-fed animals with a surrogate mother who, in all cases, appeared to care for her foster young. The effect of diurnal variation on CCPR (18) was eliminated by performing all studies at the same time of day.

It is possible that the findings described were mediated by regulatory peptides acting locally or via intermediate pathways. Different patterns of gastrointestinal hormone release in response to feeding have been described in the breast and CMF-fed human neonate (19), which may regulate both local gastrointestinal responses and more distant metabolic responses to enteral nutrition.

Maturation of the gastrointestinal tract of the guinea pig occurs late in gestation, but by full term (67 days), finger-shaped intestinal villi with well-developed microvilli are present (20). The majority of immunoglobulin transfer from mother to young occurs during late gestation via the vitelline blood vessels (21). Little transfer occurs via colostrum and intestinal closure to macromolecular uptake occurs soon after birth (22). This appears to occur independently of epithelial cell proliferation, being an "exhaustion" of the finite pinocytotic potential of the neonatal enterocyte membrane (23). An increase in the length and number of microvilli and a decrease in the high glycogen content of enterocytes (24) follows.

The weaning period in the guinea pig does not occur as abruptly as that of the rat and mouse. It is associated with a gradual transition in mucosal function from one concerned with the digestion and absorption of milk to solid food (25). In the rat and mouse weaning is not only associated with intestinal closure and abrupt changes in the mucosal hydrolase profiles (23, 26), but also with an increase in villus heights and crypt cell production rate (27). We have not followed the neonatal guinea pig through the weaning period because we were concerned in this study with the impact of initiation of milk feeding on gastrointestinal mucosal structure and function.

In the absence of significant differences in gross mucosal morphology between the two feeding groups [the differences in morphological measurements described herein and earlier (1) were due to differences in techniques of tissue fixation and preparation involved in the microdissection and standard histological methods used], we suggest that the greater CCPR seen in the CMF-fed animals was due either to an absence of modulating factors in natural milk, or a regenerative response to damage by cow's milk proteins. This led to the appearance on the villi of a population of more immature enterocytes forming an epithelium more permeable to small water-soluble markers, perhaps through poorer integrity of the mucosa at the tight junctions (28). In the rat the configuration of the tight junctions changes sufficiently with mitotic activity to permit the passage of macromolecules across the junctions (29). It would seem reasonable to suppose that such an increase in paracellular pathways could account for the greater lactulose permeability seen in association with the increased CCPR in the CMF-fed guinea pigs. Ultrastructural studies of the mucosa, with special attention on the tight junctions, are required to investigate this possibility further.

## REFERENCES

1. Weaver LT, Laker MF, Nelson R, Lucas A 1987 Milk feeding and changes in intestinal permeability and morphology in the newborn. J Pediatr Gastroenterol Nutr 6:351–358
2. Van Dongen JM, Visser WJ, Daems W Th, Galjaard H 1976 The relation between cell proliferation, differentiation, and ultrastructural development in rat intestinal epithelium. Cell Tissue Res 174:183–199
3. Weaver LT, Koritz TN, Coombs RRA 1987 Tolerance to orally induced anaphylactic sensitisation to cow's milk proteins and the patency of the intestinal mucosa in the neonatal guinea pig. Int Arch Allergy Appl Immunol 83:220–222
4. Deleted in proof.
5. Tannock IF 1965 A comparison of the relative efficiencies of various mataphase arrest agents. Exp Cell Res 47:345–356
6. Wright NA, Irwin M 1982 The kinetics of villus cell populations in the mouse small intestine. I. Normal villi: the steady state requirement. Cell Tissue Kinet 15:595–609
7. Wright NA, Appleton DR 1980 The metaphase arrest technique. A critical review. Cell Tissue Kinet 13:643–663
8. Lebenthal E, Lee PC 1983 Interactions of determinants in the ontogeny of the gastrointestinal tract: a unified concept. Pediatr Res 17:19–24
9. Koldovsky O, Thornburg W 1987 Hormones in milk. J Pediatr Gastroenterol Nutr 6:172–196
10. Read LC, Upton FM, Francis GL, Wallace JC, Dahlenberg GW, Ballard FJ 1984 Changes in the growth-promoting activity of human milk during lactation. Pediatr Res 18:133–138
11. Steimer KS, Packard R, Holden D, Klagsbrun M 1981 The serum-free growth of cultured cells in bovine colostrum and in milk obtained later in the lactation period. J Cell Biol 109:223–224
12. Toyoda S, Lee PC, Lebenthal E 1986 Interaction of epidermal growth factor with specific binding sites of enterocytes isolated from rat small intestine during development. Biochim Biophys Acta 889:295–301
13. Weaver LT, Bailey DS 1987 Effect of the lectin concanavalin A on the neonatal guinea pig gastrointestinal mucosa in vivo. J Pediatr Gastroenterol Nutr 6:445–453
14. Stern M, Pang KY, Walker WA 1984 Food proteins and gut mucosal barrier. II Differential interaction of cow's milk proteins with the mucous coat and surface membrane of adult and immature rat jejunum. Pediatr Res 18:1252–1257
15. Pang KY, Bresson JL, Walker WA 1983 Development of the gastrointestinal mucosal barrier. IV. Evidence for structural differences in microvillous membranes from newborn and adult rabbits. Biochim Biophys Acta 727:201–208
16. Bullen JJ 1976 Iron-binding proteins and other factors in milk responsible for resistance to Escherichia coli. In: Elliott K (ed) Acute Diarrhoea in Childhood. Excerpta Medica, Amsterdam, pp 149–169
17. Sprinz H 1962 Morphological response of the intestinal mucosa to enteric bacteria and its implication for sprue and Asiatic cholera. Fed Proc 21:57–64
18. Al-Nafussi AI, Wright NA 1982 Circadian rhythm in the rate of cellular proliferation and in the size of the functional compartment of mouse jejunal epithelium. Virchows Arch 40:71–79
19. Lucas A, Blackburn AM, Aynsley-Green A, Sarson DL, Adrian TE, Bloom SR 1980 Breast vs Bottle: endocrine responses are different with formula feeding. Lancet 1:1267–1269
20. Bailey DS, Cook A, McAllister G, Moss M, Mian N 1984 Structural and biochemical differentiation of the mammalian small intestine during foetal development. J Cell Sci 72:195–212
21. Barnes JM 1959 Antitoxin transfer from mother to foetus in the guinea-pig. J Pathol Bacteriol 77:371–380
22. Lecce JG, Broughton CW 1973 Cessation of uptake of macromolecules by neonatal guinea pig, hamster and rabbit intestinal epithelium (closure) and transport into blood. J Nutr 103:744–750
23. Rundell JO, Lecce JG 1972 Independence of epithelial cell turnover from cessation of absorption of macromolecules (closure) in the neonatal mouse, rabbit, hamster and guinea pig. Biol Neonate 19:304–326
24. Merrill TG, Sprinz H, Tousimis AJ 1967 Changes in intestinal absorptive cells during maturation: an electron microscopic study of prenatal, postnatal, and adult guinea pig ileum. J Ultrastruct Res 19:304–326
25. Koldovsky O, Heringova A, Jirsova V, Chytil F, Hoskova J 1966 Postnatal changes in beta-galactosidase activity in the jejunum and ileum of mice, rabbits, and guinea pigs. Can J Biochem 44:523–527
26. Henning SJ 1981 Postnatal development: coordination of feeding, digestion and metabolism. Am J Physiol 241:199–214
27. Al-Nafussi, Wright NA 1982 Cell kinetics in the mouse small intestine during immediate postnatal life. Virchows Arch 40:51–62
28. Mora-Galindo J 1986 Maturation of tight junctions in guinea-pig cecal epithelium. Cell Tissue Res 242:169–175
29. Tice LW, Carter RL, Cahil MB 1979 Changes in tight junctions of rat intestinal crypt cells associated with changes in their mitotic activity. Tissue Cell 11:293–316