The influence of the gut microflora and of dietary fibre on epithelial cell migration in the chick intestine

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(*Received* 1 *November* 1976 – *Accepted* 4 *April* 1977)

1. The renewal of the intestinal mucosal epithelium has been investigated in germ-free and conventional chicks given a practical chick diet and a low- and high-fibre diet, using tritiated thymidine to label the nuclei of mucosal cells undergoing division.

2. Villus height, crypt depth and number of mitoses in the crypt were generally, although not always significantly, greater for conventional chicks than for germ-free chicks at all sites investigated in the intestine, and all became progressively lower from the upper intestine to the lower intestine.

3. There was a linear relationship between the height of the radioactively labelled mucosal cells on the villus and the period after injection, and the rates of epithelial cell migration were higher for conventional than for germ-free chicks, the relative differences being greater in the lower intestine than the upper intestine.

4. The inclusion of wheat bran as a source of dietary fibre had no effect on the rate of epithelial renewal, either in germ-free or conventional birds.

In the intestine epithelial cells are produced in the crypts and travel along the villi to be shed from the villi tips. This process has been shown to be subject to many influences, including the nutritional status of the animal and the presence of an intestinal microflora (e.g. Leblond & Messier, 1958; Abrams, Bauer & Sprinz, 1963; Cook & Bird, 1973; Moon & Joel, 1975). Clearly this continuous replacement of the villus epithelium makes demands on the nutritional resources of the animal. In the work described here the possible influences on renewal of the epithelial mucosal cells of the intestinal flora and of dietary fibre, separately and together, were investigated. In Expt 1, germ-free and conventional chicks eating a practical-type chick mash were used to evaluate the effect of the intestinal microflora. In Expt 2, a purified, low-residue diet, with or without added fibre, was given to both germ-free and conventional chicks to study any interaction between fibre and the gut microflora. The cellular migration was followed by injecting the birds with tritiated thymidine, which became permanently incorporated in the nuclear material of cells undergoing mitosis so that their subsequent position could be determined by autoradiography.

MATERIALS AND METHODS

Chicks

Rhode Island Red × Light Sussex cross chicks were used. Eggs from a specified pathogen-free flock maintained at this Institute were incubated for 18 d in a commercial incubator and then disinfected by spraying with peracetic acid solution (Harrison, 1969). Half the eggs were transferred to Gustafsson stainless-steel isolators (Gustafsson, 1959) and maintained germ-free and the other remaining eggs were replaced in the incubator. After hatching, the germ-free chicks were housed in groups of four birds in stainless-steel cages with mesh floors inside the isolators while the conventional chicks were kept in rooms where the physical environment could be maintained to match that present within the isolators. Males and females were distributed evenly among the experimental groups as far as possible. The continued microbial sterility of the birds within the isolators was checked at intervals (Fuller, 1968).

Diets

In Expt 1 the diet contained (g/kg): maize meal 378·0, barley meal 200·0, soya-bean meal 345·0, dried-grass meal 30·0, bone meal 15·0, limestone flour 10·0, sodium chloride 6·72, vitamin supplement 5·0, maize oil 10·0, $MnSO_4.4H_2O$ 0·28. Cholecalciferol was dissolved in the maize oil to provide 16 μg/kg diet. Rovimix A500 (Roche Products, Welwyn Garden City, Herts) was added to supply 2 mg retinol/kg. The vitamin supplement provided (mg/kg diet): riboflavin 7·7, nicotinic acid 55, biotin 0·22, pteroylmonoglutamic acid 0·83, thiamin hydrochloride 3·3, pyridoxine hydrochloride 4·4, calcium pantothenate 16·5, cyanocobalamin 0·02.

In Expt 2, the low-residue diet contained (g/kg): maize starch 596·5, casein 180, gelatin 100, salt mixture 60, L-cystine 3·0, choline chloride 1·5, *myo*-inositol 1·0, vitamin supplement 8·0, maize oil 50. The salt mixture supplied (/kg diet): $CaCO_3$ 17·1 g, KH_2PO_4 13·3 g, $CaHPO_4.2H_2O$ 17·1 g, NaCl 8·67 g, $MnSO_4.4H_2O$ 270 mg, KI 37 mg, $CuSO_4.5H_2O$ 16 mg, $ZnSO_4.7H_2O$ 130 mg, $MgSO_4.H_2O$ 2·67 g, $FeSO_4.7H_2O$ 670 mg. Fat-soluble vitamins dissolved in maize oil provided (mg/kg diet): cholecalciferol 0·16, menaphthone 20, α-tocopheryl acetate 40. Rovimix A500 was added to provide 20 mg retinol/kg diet. The vitamin supplement provided (mg/kg diet): biotin 0·8, pteroylmonoglutamic acid 6·0, thiamin hydrochloride 12·0, pyridoxine hydrochloride 16·0, riboflavin 24·0, calcium pantothenate 60·0, nicotinic acid 160·0, cyanocabalamin 0·08. For the fibre-containing diet, coarse unprocessed wheat bran was added at the rate of 100 g/kg at the expense of maize starch.

After mixing, all the diets were granulated, packed into plastic bags and sterilized by gamma radiation at 5 Mrad. Vitamin supplements to the purified diets were high to compensate for possible destruction of activity during irradiation.

Experimental

In Expt 1 eight chicks were maintained in each environment, all eating the practical chick mash *ad lib.* In Expt 2 the diet without bran and the diet with bran were each given *ad lib.* to eight chicks in both germ-free and the conventional environments.

When the chicks were 4 weeks old each was injected intraperitoneally with approximately 250 μCi [6-^{3}H]thymidine in sterilized aqueous solution at a specific activity of 2 Ci/mmol and a concentration of radioactivity of 1 mCi/ml (The Radiochemical Centre, Amersham, Bucks). Samples were taken at 24, 48, 72 and 96 h after injection in Expt 1 and at 12, 24, 36, 48, 60, 72, 84, and 96 h in Expt 2; the germ-free birds being removed from the isolators immediately before sampling. The chicks were anaesthetized with diethyl ether and the alimentary tract was exposed. In Expt 1 lengths of 10–20 mm were removed from the following regions: the middle of the proximal limb of the duodenal loop and at three points in the ileum; immediately distal to the entry of the bile ducts, immediately proximal to the yolk stalk and approximately 100 mm proximal to the ileo-caecal junction. In Expt 2, lengths of 10–20 mm were also removed from the middle of the colon. We have referred to the whole of the postduodenal small intestine as the ileum since beyond the duodenum there are no clear histological distinctions between different regions of the chick small intestine. The birds were then killed. The gut segments were cut open along their length, pinned flat on a cork strip and fixed in ethanol (700 ml/l) – formalin – glacial acetic acid (20:2:1, by vol.). The dehydrated specimens were embedded in paraffin and sectioned at 4 μm. Autoradiography was carried out by the method of Rogers (1969). The sections were dipped in 'nuclear research' emulsion (Type K2, Ilford Ltd, Ilford, Essex) diluted with water and glycerol, exposed for 4 weeks at $-20°$ and developed using Amidol (2,4-diaminophenol hydrochloride; BDH Ltd, Poole, Dorset) developer prepared according to Rogers (1969). The autoradiographs were then stained with haematoxylin.

All epithelial dimensions were measured in terms of numbers of cells since the convolutions of the villus surfaces and possible distortion during sample preparation made linear measurements impossible. In both experiments longitudinal sections of intact villi were examined to determine the position of the 'leading edge' of the labelled cells, as indicated by the presence of silver grains over their nuclei. In Expt 1 twenty replicate readings of epithelial dimensions and the position of the 'leading edge' of the labelled cells were made for each intestinal sampling site, but in Expt 2 this was reduced to seven replicate readings as statistical treatment had shown that the higher number did not result in a worthwhile improvement in precision. The readings were then averaged to give one value/site per chick as preliminary calculation showed that for a given site variation 'between replicates-within chicks' was markedly smaller than variation between chicks. The relationship between height of labelled cells on the villus and period after injection was investigated by regression analysis and the regression coefficients for different intestinal sites, the two diets and the two environments were compared. In Expt 1 the following morphological characteristics were also measured in the histological specimens: (1) height of the villi, (2) depth of the crypts, (3) the mitotic index, i.e. the number of cells in process of division within a crypt. Duplicate measurements were made on two sections at each site for each chick and the mean values were compared for effects of environment and site.

RESULTS

Morphological observations

The measurements made of the epithelial morphology are given in Table 1. At all sites examined the villi of germ-free birds were more regular, slender and finger-like than those of conventional chicks. The average villus height for germ-free chicks was smaller than that for conventional chicks. However, this difference was not significant in the duodenum and was non-existent in the upper ileum. Villus height, and the ratio, height:width, decreased sharply from the duodenum to the lower ileum in both environments. Crypt depth diminished similarly along the gut in both germ-free and conventional chicks, but the depth was always greater in conventional birds. There was a statistical interaction between sites and environments ($P < 0{\cdot}01$) and inspection of Table 1 suggests that this may have arisen because the differences in epithelial dimensions between samples from conventional chicks and germ-free chicks were smaller in the duodenum than at other intestinal sites sampled. There were more mitoses in conventional chicks than in germ-free chicks, although the effect of environment did not reach significance at the beginning and the end of the ileum.

Epithelial cell migration

The relationship between the distance along the villus that the radioactively labelled cells had migrated (numbers of cells from the bottoms of the crypts) and the period (h) after the administration of the radioactive material was significant for all sites and treatments in both experiments ($P < 0{\cdot}05$). The relationship was linear except for the upper ileum from conventional chicks in Expt 1, when there was evidence of curvature such that cell movement accelerated with the period after injection. Linear regression coefficients were calculated for each site, environment and diet (Tables 2 and 3). Typical results for one chick for each treatment are shown in Fig. 1.

In Table 2, the linear regression coefficients are compared for each site and environment in the first experiment, when the chicks were given a practical diet. Standard errors of differences between regression coefficients for the two environments for a particular site were based on the error mean square 'chicks within times and environments' pooled for the

Table 1. *Villus height and crypt depth (numbers of cells) and mitotic index (number of dividing cells/crypt) at four sites in the intestine in sixteen germ-free and sixteen conventional chicks aged 4 weeks, given a practical diet**

Site	Environment: Germ-free	Environment: Conventional	Difference between environments	SE of difference (14 df)	Statistical significance of difference: $P <$
		Villus height			
Duodenum: proximal limb	249	279	30	16	> 0·05
Ileum: Distal to bile ducts	221	219	−2	17	> 0·05
Proximal to yolk stalk	114	137	23	7	0·01
Proximal to ileo-caecal junction	101	123	22	8	0·05
SE of difference between sites (21 df)	11	15			
Mean of four sites	171	190	18	6	0·05
		Crypt depth			
Duodenum: proximal limb	43·2	49·6	6·3	1·7	0·01
Ileum: Distal to bile ducts	34·1	44·4	10·2	1·6	0·001
Proximal to yolk stalk	29·3	37·8	8·5	1·6	0·01
Proximal to ileo-caecal junction	24·1	34·2	10·1	1·2	0·001
SE of difference between sites (21 df)	0·6	1·1			
Mean of four sites	32·7	41·5	8·8	1·3	0·001
		Mitotic index			
Duodenum: Proximal limb	0·94	2·06	1·12	0·43	0·05
Ileum: Distal to bile ducts	1·25	2·12	0·87	0·41	> 0·05
Proximal to yolk stalk	0·69	2·37	1·68	0·23	0·001
Proximal to ileo-caecal junction	0·50	1·44	0·94	0·46	> 0·05
SE of difference between sites (21 df)	0·33	0·41			
Mean of four sites	0·84	2·00	1·16	0·23	0·001

* For details, see p. 92.

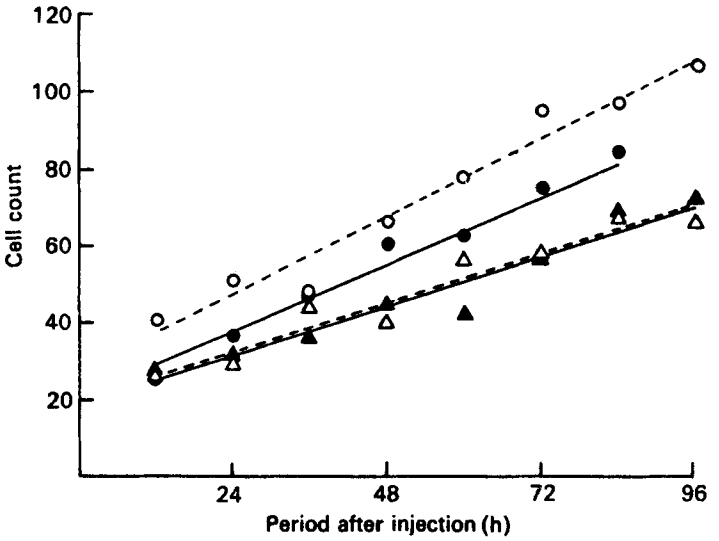

Fig. 1. Position of the radioactively labelled cells on the villus (numbers of cells from the bottom of the crypt) at different intervals after the administration of tritiated thymidine (h), in the lower ileum of germ-free (▲—▲, diet without bran; △---△, diet with bran) and conventional (●—●, diet without bran; ○---○, diet with bran) chicks aged 4 weeks. The calculated regression lines of cell count *v* period after injection are drawn. Points represent values for one chick.

Regression lines are calculated as:

Environment	Diet	
Conventional	Without bran	$y = 20{\cdot}6+0{\cdot}73t$
	With bran	$y = 27{\cdot}5+0{\cdot}84t$
Germ-free	Without bran	$y = 18{\cdot}0+0{\cdot}55t$
	With bran	$y = 20{\cdot}5+0{\cdot}52t$

where y is the cell count and t is the period after injection. For details of treatments, see p. 92.

Table 2. *Relationships between height of radioactively labelled cells on villus (numbers of cells from the bottom of the crypt) and period after injection of tritiated thymidine (h), and statistical analyses for groups of chicks aged 4 weeks, given a practical diet* and maintained in a germ-free or conventional environment*

(Values for eight chicks in each environment, two chicks analysed at each of four intervals after injection)

Site	Regression coefficients: Environment: Germ-free	Regression coefficients: Environment: Conventional	Differences between environments	SE of difference (8 df)	Statistical significance of difference: $P <$
Duodenum: proximal limb	1·69	2·12	0·43	0·34	> 0·05
Ileum: Distal to bile ducts	1·42	2·13	0·71	0·19	0·01
Proximal to yolk stalk	0·83	1·67	0·84	0·12	0·001
Proximal to ileo-caecal junction	0·47	1·25	0·78	0·09	0·001
SE of differences between sites (12 df)	0·17	0·09			
Mean of four sites	1·10	1·79	0·69	0·17	

* For details, see p. 92.

Table 3. *Relationships between height of radioactively labelled cells on villus (numbers of cells from the bottom of the crypt) and period after injection of tritiated thymidine (h), and statistical analyses for groups of chicks aged 4 weeks, given a low-residue diet* or the same diet with 100 g/kg unprocessed wheat bran, and maintained in a germ-free or conventional environment*

(Values for eight chicks for each diet and for each environment)

Site	Environment: Germ-free, Diet without bran	Environment: Germ-free, Diet with bran	Environment: Conventional, Diet without bran	Environment: Conventional, Diet with bran	Differences in regression coefficients: Between environments	Differences in regression coefficients: Between diets	SE of differences (23 df)	Statistical significance of differences between regression values: $P <$ Between environments	Statistical significance of differences between regression values: $P <$ Between diets
Duodenum: proximal limb	1·33	1·30	1·62	1·48	0·24	−0·17	0·14	> 0·05	> 0·05
Ileum: Distal to bile ducts	1·10	1·23	1·55	1·59	0·40	0·08	0·10	0·001	> 0·05
Proximal to yolk stalk	0·95	0·79	1·30	1·43	0·50	−0·02	0·08	0·001	> 0·05
Proximal to ileo-caecal junction	0·55	0·52	0·73	0·84	0·25	0·04	0·06	0·001	> 0·05
Large intestine	0·48	0·44	0·52	0·53	0·06	−0·02	0·04	> 0·05	> 0·05
SE of differences between sites (48 df)	0·09	0·09	0·14†	0·17†					
Mean of five sites	0·88	0·86	1·14	1·17	0·28	0·00	0·05	0·001	> 0·05

* Diet without bran; for details, see p. 92.

† 44 df due to missing value.

two environments (8 df). The errors are reported separately for each site because of heterogeneity of error between sites. A similar type of error was used to compare regression coefficients of the two environments averaged over the four sites. The standard errors of the differences between regression coefficients at different sites within a given environment were derived from the error mean square 'sites × chicks within times' (12 df). The slope of the regression line, which represented the average speed of cell movement along the villus, was greater for conventional chicks in all cases, although statistical significance was not established for the duodenum. The speed of migration of epithelial cells along the villus generally became progressively slower along the small intestine from the duodenum to the lower ileum, although differences were not established with confidence between the duodenum and upper ileum in either environment or between the mid- and lower ileum for germ-free chicks.

A comparison of the regression coefficients for the second experiment is presented in Table 3. The standard errors of the differences 'between environments' and 'between diets' for a particular site are based on the combined residual mean square of the four regressions at that site (23 df, because of a missing value at 96 h for conventional chicks given the diet with bran). As in Table 2, heterogeneity of error between sites led to the separate reporting of errors. A similar type of error was used to compare environments and diets averaged over the five sites. The standard errors for comparing regression coefficients between sites were based on the pooled mean square 'sites × deviations from regression within environment' (48 df for germ-free chicks, and 44 df for conventional birds (due to the missing value)). As in Expt 1 the rates at which epithelial cells traversed the villus were greater for conventional chicks than for germ-free chicks, although for the duodenum and large intestine clear differences were not established. Again, the rate of epithelial cell movement decreased from the duodenum to the large intestine. The presence of fibre in the diet with bran did not affect the rate of epithelial cell migration. Extrapolation of the regression line to zero time gave a positive intercept in all cases. In the lower ileum and large intestine of conventional chicks, although the radioactively labelled cells travelled along the villus at similar rates whether or not the diet contained bran, there was a significant difference between the intercepts ($P < 0.001$) such that the radioactive label was further along the villus when the diet contained bran.

DISCUSSION

The comparisons between germ-free and conventional chicks establish clearly that the presence of a gut microflora promoted greater mitotic activity, faster migration and a more rapid turnover of epithelial cells in the intestinal mucosa. The villus height, crypt depth and mitotic index were generally lower in the germ-free intestine, where the epithelium was more regular, and the villi more slender and finger-shaped, than in the corresponding conventional tissue. Our observations are in line with those of earlier studies on the lower ileum of the mouse (Abrams *et al.* 1963), the duodenum of the 1-week-old Leghorn chick (Cook & Bird, 1973) and the small intestine of the rat (Meslin, Sacquet & Guenet, 1973; Meslin, Sacquet & Raibaud, 1974).

In our studies the villus height, crypt depth, mitotic index and cell turnover rate decreased progressively along the intestine from duodenum to lower ileum in both environments. With the exception of crypt depth, the differences between germ-free and conventional birds were less apparent in the region of the duodenum, where the bacterial population is comparatively low, and became more marked towards the terminal ileum. Meslin and his co-workers (Meslin *et al.* 1973, 1974) sampled the small intestines of 3-month-old rats at positions 0·1, 0·5 and 0·9 of the lengths. They also found that villus height and crypt depth decreased towards the distal end of the intestine and that the difference between germ-free and

conventional animals was not significant in the duodenal region. However, they reported little difference in mitotic index between one site and another.

The normal condition of the intestinal epithelium of conventional animals has been described as 'physiological inflammation' by Sprinz (1962), but the mode of action of the gut micro-organisms on the epithelium is a matter of some debate. Ranken, Wilson & Bealmear (1971) suggested from work on mice that cholic acid, formed by bacterial deconjugation of biliary taurocholic acid, regulates epithelial cell renewal, but Meslin *et al.* (1974) concluded that free cholic acid was not the only factor involved since they observed an increased rate of epithelial cell renewal when germ-free rats were associated with micro-organisms that do not deconjugate bile salts. Lignin is capable of sequestering bile acids (Eastwood & Hamilton, 1968). The effect on epithelial cell renewal of including wheat bran at a concentration that provided about 45 g dietary fibre/kg low-residue diet was negligible, lending support to the suggestion of Meslin *et al.* (1974) that factors other than bile acids are concerned in its regulation. Cereal fibre has hydrophilic properties and its presence in gut contents increases their bulk and rate of passage through the gastrointestinal tract. Apparently neither of these characteristics influences epithelial cell turnover, which was virtually unaltered by inclusion in the diet of an appreciable proportion of fibrous material. It is equally clear that, as far as renewal of the intestinal epithelial cells is concerned, the consumption of dietary fibre in the form of wheat bran makes no additional demands on the nutritional resources of the animal.

The finding that cell migration regression lines do not pass through the origin is in agreement with the findings of other workers and must reflect the fact that cellular proliferation within the crypt takes place over an area and not at a single point at the base of the crypt. The larger positive intercept in the lower gut of conventional chicks given the diet with bran may be interpreted as meaning that the area of proliferation is increased by the joint action of dietary fibre in the form of bran and bacteria, or that there are differences in the rate of cell movement before 12 h, when no observations were made. It is even possible that bran may increase the migration rate to an extent not detectable from these results, since statistical tests for differences in slope are less sensitive than for differences in intercept.

We thank Mrs M. Payne and Mr P. Seabrook for technical assistance and Mr J. P. Fordham for care and maintenance of the germ-free chicks. We are grateful to Mr D. R. Westgarth and Dr D. Hewitt for valuable help with the design of the experiments and to Mrs P. Buckingham for statistical calculations.

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Printed in Great Britain