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Development of the pattern of cell renewal in the crypt–villus unit of chimaeric mouse small intestine

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Summary

We have previously shown that the epithelium of each adult intestinal crypt in chimaeric mice is derived from a single progenitor cell. Whether the crypts are monoclonal from the outset – that is, are formed by the proliferation of a single cell – or whether their formation is initiated by several cells was not known. Here we report that many crypts contain cells of both chimaeric genotypes in the neonatal period indicating a polyclonal origin at this stage of morphogenesis. The cellular organization of the early neonatal crypt is therefore different from that of the adult crypt, which includes a zone of 'anchored' stem cells above the crypt base. Within 2 weeks, however, the crypt progenitor cell and its descendants displace all other cells from the crypt and the crypt attains monoclonality. The distribution of enterocytes on chimaeric villi in the neonate shows a mottled pattern of mosaicism which is progressively replaced by coherent sheets of cells from the crypts, and within two weeks the orderly adult clonal pattern is established.

Key words: chimaera, mouse intestine, crypt, killus, monoclonal origin, polyclonal origin, *Dolichos biflorus* agglutinin.

Introduction

The epithelium of the adult small intestine is anatomically highly organized into villi (finger-like projections into the lumen of the gut) and crypts (tubular invaginations surrounding villi). Cells proliferate within the crypts, migrate up the villi and are shed. In mice, villi are formed shortly before birth by the coalescence of clefts which appear between the cells of the multilayered intestinal epithelium. Into these groups of epithelial cells the core of each villus develops by upward growth of the mesenchyme. By the time of birth, the intestinal epithelium is a sheet of single-cell thickness. The crypts develop from the flat intervillus epithelium during the few days after birth (Mathan *et al.* 1976). Morphologically, the crypts are first recognizable as shallow depressions in the epithelium, but it is not clear whether their formation is initiated by the proliferation of a single cell or by the proliferation or folding of the epithelium which initially involves several cells. We have investigated the development of crypts and villi in the neonatal period using C57BL/6J Lac (B6) ↔ SWR mouse embryo aggregation chimaeras, a combination that has been used in our previous studies of the adult mouse intestine (Ponder *et al.* 1985*b*; Schmidt *et al.* 1985). The visual mosaic marker in these chimaeras is based on the presence in B6 but not in SWR intestinal epithelium of binding sites for the lectin *Dolichos biflorus* agglutinin (DBA) (Ponder *et al.* 1985*a*).

Materials and methods

Mice

B6 and SWR mice were obtained from Olac Ltd. (Bicester UK). Ten chimaeras were obtained by aggregation of 4- to 8-cell embryos according to methods described by Mintz (1971). B6 × DBA/2 Lac F_1 hybrids were used as foster mothers. The chimaeras were analysed at postnatal days 2 (two animals), 6 (two), 10 (three) and 14 (three).

Preparation of crypt sections

Serial sections of intestine were obtained as follows: 2 cm segments of duodenum were placed onto white card for support. They were then fixed in methacarn overnight at 4 °C, followed by 70 % ethanol fixation and paraffin embedding. The supporting card was removed before making paraffin blocks. Serial longitudinal sections resulted in

several slides per specimen where tangential cuts revealed areas containing crypts in complete circular cross-sections. A complete circular cross-section was required to distinguish crypts from folds in the intervillus epithelium.

Preparation of villi

The duodenal parts of the intestine were dissected out, cut open along the mesentery and pinned out in cold phosphate-buffered saline, pH 7·3, on wax-based Petri dishes with the luminal surface uppermost. Gut contents were removed by gentle flushing. The preparations were fixed in 10 % buffered formalin for 1 h; they were washed in PBS (several changes). To remove mucin, the preparations were incubated for 1 h in 20 mM-DL-Dithiothreitol (DTT, Sigma, Poole, UK) made up in 4 ml ethanol, 14 ml NaCl, 2 ml of 150 mM-Tris buffer (pH 8·2). The intestinal sheets were subsequently stored in 10 % buffered formalin.

Analysis of mixed crypts

Using longitudinal histological sections of chimaeric neonatal duodenum, all crypts along both sides of clearly defined patch boundaries were counted. Owing to the shallowness of newly formed crypts, the total number of scorable crypts was lower in the 2- and 6-day-old specimens than the 10- and 14-day-old ones; hence the lowest score of a total of 29 crypts was obtained from a 2-day-old specimen. The data were analysed by regression analysis.

Staining

DBA was purchased from Sigma. Peroxidase conjugates were prepared by the periodate method (for details see Ponder & Wilkinson, 1983). 10 mg of horseradish peroxidase (Miles, Slough, UK) were conjugated with 10 mg of lectin in the presence of a 2 % concentration of the specific inhibiting sugar, N-acetyl galactosamine (Sigma). A typical preparation yielded 30 ml (stored in 1 ml samples at 4 °C) of DBA–peroxidase conjugate and was used at a dilution of 1:75.

The fixed preparations of intestine were incubated for 30 min in 0·1 % phenylhydrazine HCl in PBS, pH 7·3 to block endogenous peroxidase and then incubated for one hour (sections) or overnight in DBA–peroxidase conjugate 1:75 in PBS containing 0·5 % bovine serum albumin (Sigma) (PBS–BSA). Between each incubation the preparations were carefully washed in PBS–BSA. The peroxidase was subsequently demonstrated using 3′3′diaminobenzidine (DAB) (Sigma) as a substrate yielding a brown reaction product (Ponder & Wilkinson, 1983).

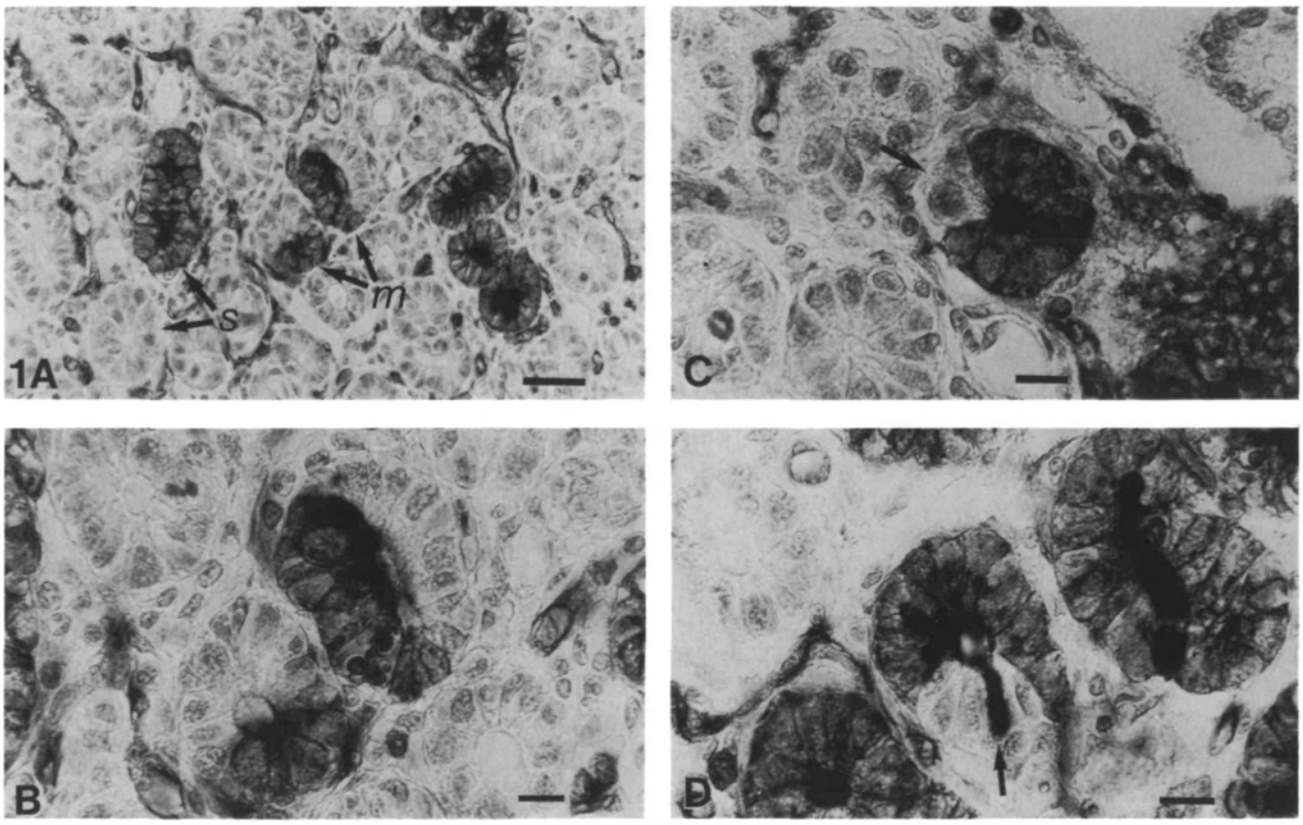

Fig. 1. Cross-section through crypts in neonatal duodenum. B6 → SWR chimaera; 6 days old. 4 μm paraffin section counterstained with haemalum. The B6 component (black) is stained by DBA–peroxidase, the SWR component is not stained. *m*, mixed crypts: the epithelium contains cells of each genotype. *s*, unmixed B6 or SWR crypts. (A) The contribution of the two components to the mixed crypts is balanced; shown in B at higher magnification. (C) The SWR component is in the minority (2 cells; arrow). (D) The SWR component is separated by a cell of B6 type (arrow). Scale bar equals 30 μm (A) or 10 μm (B–D).

Controls

In B6 control mice, all the epithelial cells in each crypt or on each villus were stained by DBA–peroxidase; and epithelial staining was not found in comparable sections of SWR intestine.

Photography

Individual villi were isolated with the aid of an Eye Blade (Beaver KB-225-06, Downs Surgical Ltd, Mitcham, Surrey). Villi were photographed on a Zeiss photomicroscope, histological sections on a Zeiss Axiophot photomicroscope, using Ilford Pan F 50 ASA film.

Results

In the chimaeric system, crypts inevitably are composed of cells of only one genotype when they arise *within* a patch whether being from one or from several cells. Mixed crypts are only detected when they form at the boundary between patches of different genotype. At day 2 after birth, 50 % of the crypts at the borders between chimaeric patches were of mixed type (Fig. 1). The contribution of the two components to a mixed crypt in sections included all possible cases, from completely balanced to only one cell being of either B6 or SWR genotype. Serial sections were not obtained and we have therefore no information about the two-dimensional shapes of patches. The proportion of mixed crypts at patch boundaries steadily decreased at days 6 and 10, and by day 14 the large majority of crypts was monoclonal (Fig. 2, Table 1).

In whole mounts of day-2 neonatal intestine, villi appeared mottled with no indication of cells occurring in orientated coherent sheets (Fig. 3A). By day 6, however, occasional straight ribbons of cells were observed. In chimaeras with relatively equal proportions of the two parental components the great majority of villi were mottled (Fig. 3B). On day 10, stripes of variable width and irregularly arranged patches were found (Fig. 3C). By day 14, the stripy patterns demonstrated in whole-mount preparations of chimaeric adult villi (Schmidt *et al.* 1985) prevailed (Fig. 3D); patches of isolated groups of cells (i.e. surrounded by the other genotype) were rare and always confined to the apical part of villi.

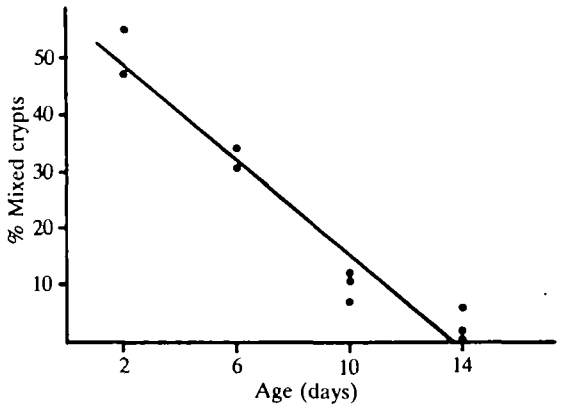

Fig. 2. Frequency of mixed crypts at patch boundaries. Each datum point represents one chimaera. 29–200 crypts were counted for each chimaera; the total number of crypts counted was 1350. Regression analysis showed a highly significant ($P < 0{\cdot}01$) linear negative correlation of the percentage of mixed crypts (mean values) against time (days). The regression line $Y = -4{\cdot}15525X + 57{\cdot}3025$ is shown.

Discussion

Potential crypt progenitor cells cannot be identified in the intervillus epithelium. The high ^{3}HTdR labelling indices of intervillus areas reported in cell kinetic studies (O'Connor, 1966) suggest that it may not be uncommon for more than one proliferative cell to be incorporated in a single nascent crypt. The gradual disappearance of mixed crypts during the first 2 weeks after birth and the observation that adult crypts are always 'monoclonal' means that the progeny of only one of these proliferative cells is finally retained in each single fully formed crypt. The apparent initial displacement of all other cells from the crypt and their replacement by the progeny of only one progenitor cell has implications regarding the establishment of the stem cell zone. Models of crypt organization suggest that the fourth or fifth cell position from the base of the crypt provides a 'stem cell zone' either as a ring or a series of focal points of 16 'anchored' stem cells (Potten & Hendry, 1983); other models postulate a zone of stability (anchorage) of up to 14 scattered focal points within the first four cell positions of a crypt (Bjerknes & Cheng, 1981*a*,*b*). Our data imply that such stem cells can only retain a fixed position *after* the 'purification' process is completed,

Table 1. *Analysis of variance of the data shown in Fig. 2*

Source of variation	Sum of squares (*ss*)	Degrees of freedom (*df*)	Mean square (*ss*/*df*)	Variance ratios (*F*)	Level of significance (*P*)
Regression	1381·29	1	1381·29	54·2	<0·01
Remainder	50·97	2	25·49		
Total	1432·26	3			

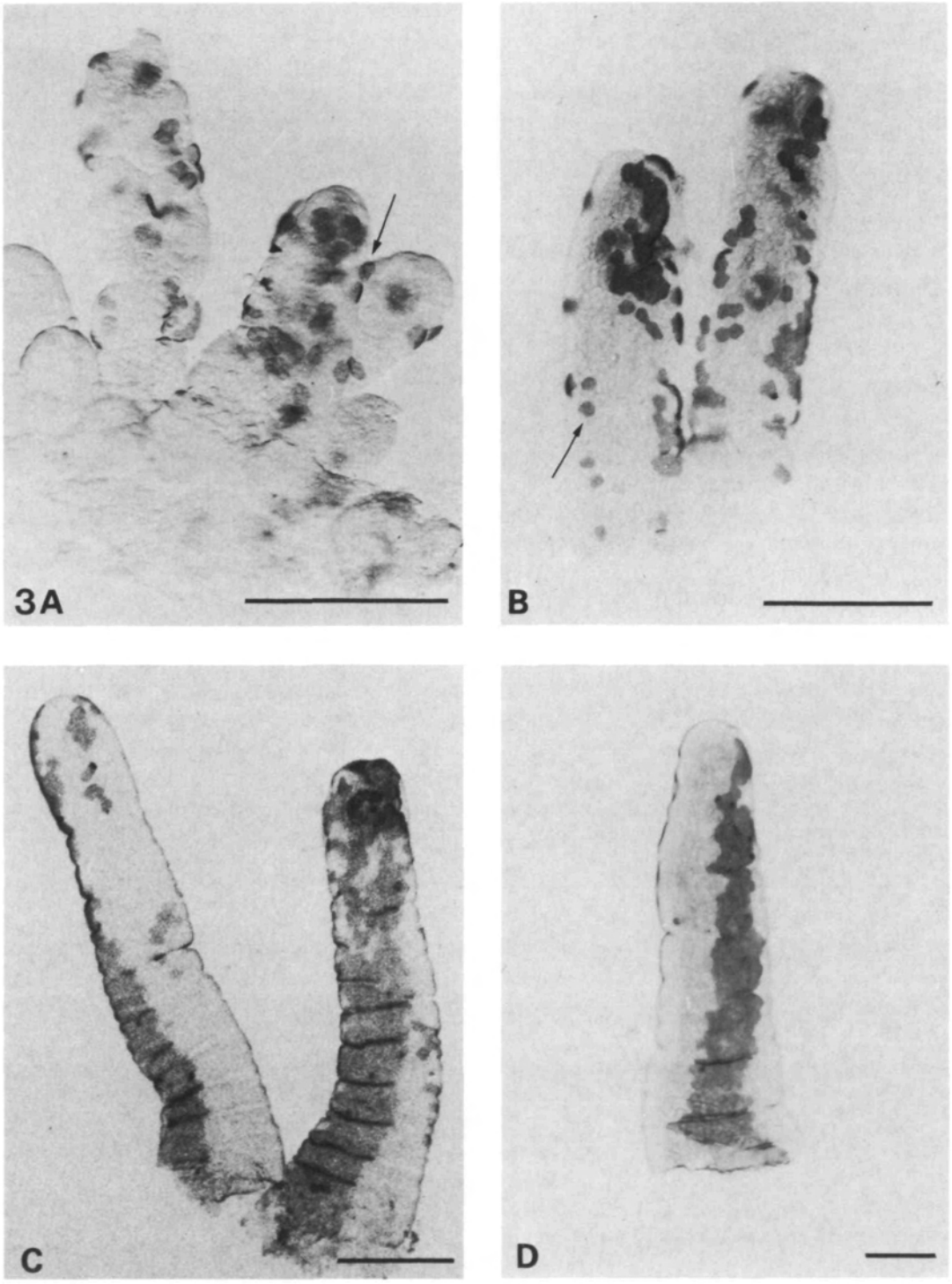

Fig. 3. Mosaic cellular patterns of chimaeric B6 ↔ SWR neonatal duodenal villi. (A) 2-day-old chimaera. (B) 6-day-old chimaera. The smallest isolated patches generally comprise two or more cells (arrows). Villi are extensively mottled. (C) 10-day-old chimaera. Straight coherent cell sheets are present; however, a mottled distribution of cells is still found towards the tip of the now tapered villi. (D) 14-day-old chimaera, showing the regular striped cellular pattern of adult chimaeric villi. Bar, 0·1 mm.

which occurs between crypt formation and day 14. The 'purification' process can be explained by regarding the development of the crypt from a stochastic point of view where the self-renewal probability of proliferative cells declines with increasing cell position (Potten & Hendry, 1983). Hence, in the newly

formed shallow crypt, the cell at the very base of the crypt alone is retained, and only after a critical increase in the depth of the crypt (Al-Nafussi & Wright, 1982) do cells above the base attain a sufficiently high probability of self renewal. The establishment of the stem cell organization within 2 weeks coincides with, and may account for, the marked increase in the cell production rate (metaphases in the crypt) from the second to the third week of life (Al-Nafussi & Wright, 1982).

Crypts continue to increase considerably in numbers after the disappearance of mixed crypts in the second week: during the third week there is an increase of about 77 % (Obuoforibo & Martin, 1977). The later rises in crypt numbers occur by means of a different process to that of earlier stages, probably primarily involving crypt fission (St. Clair & Osborne, 1985); in the first week of life only about 5 % of crypts show evidence of fission but an increase to about 30 % occurs during the second and third week (Cheng & Bjerknes, 1985). Although crypt fission may contribute to the process of crypt purification, it is unlikely to play a major part for the majority of crypts have attained monoclonality before the time when crypt fission becomes sufficiently frequent.

Data on cell kinetics in the neonatal intestinal epithelium suggest that neonatal villus enterocytes have a longer life span than adult enterocytes. Rundell & Lecce (1972) reported an epithelial replacement time of 7 days for 3-day-old mice, and Al-Nafussi & Wright (1982) calculated a 'villus transit time' of about 260 h ± 40 (i.e. 11–14 days) for mice analysed in the first week of life. These data agree well with our observation that the stripy pattern of coherent clones of cells (Fig. 3D), characteristic of the adult intestinal epithelium, takes about 14 days to become established after birth. Smith & Jarvis (1978) found that fetal-type pig enterocytes swell after short contact with distilled water, whereas adult type cells do not. Using this method Smith & Peacock (1980) demonstrated an eventual clean replacement of fetal-type by adult-type enterocytes, although initially some adult cells may bypass individual fetal-type cells thereby leaving behind small patches. The patterns revealed on chimaeric mouse villi at day 10 (Fig. 3C) are consistent with this view of enterocyte replacement in the neonate.

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