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Clonal analysis of intestinal crypt populations in mouse aggregation chimaeras

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SUMMARY

The epithelium of each individual intestinal crypt in adult mouse aggregation chimaeras is composed of cells of a single parental genotype (Ponder *et al.* 1985). Using a carbohydrate polymorphism recognized by *Dolichos biflorus* agglutinin as a strain-specific marker on entire sheets of intestinal mucosa, we have analysed the two-dimensional mosaic patterns of patches of the chimaeric intestinal crypt population. The relative proportions of each genotype varied greatly along the length of any one intestine. In chimaeras with highly unbalanced proportions, the minority component occurred as discrete patches. Patches of single or a few crypts were most frequent, but a smaller number of much larger patches was always present. The size frequency distribution of discrete patches was highly concave and departed significantly from a geometric distribution (a model for non-differential proliferation), but fitted the more skewed negative binomial model. The data are consistent with the interpretation that most progenitor crypts never or rarely divide, while a minority proliferate to a greater extent. We discuss ways in which our system could be analysed further to examine this interpretation. Our results also support Whitten's (1978) conclusion from a computer simulation that the mean patch size, as it has previously been used in statistical analyses of chimaeric tissue, 'is not a reliable statistic on which to judge mosaicism'.

INTRODUCTION

Mammalian chimaeras are experimental systems suitable for assessing the contributions of cell mingling and coherent growth in development. Groups of contiguous cells that are descended from a single progenitor cell through previous divisions are defined as coherent clones (Nesbitt, 1974). Such clones may be identified in chimaeric tissues with strain-specific histochemical markers (Ponder & Wilkinson, 1983; Ponder, Wilkinson & Wood, 1983), and then appear as patches of like genotype. Adjacent coherent clones of like genotype will, of course, appear as a single patch (see Materials and Methods).

Key words: Mouse, chimaera, intestinal crypts, clones, proliferation, *Dolichos biflorus* agglutinin.

The results from several previous quantitative studies of chimaeric tissues – liver, retina and cerebellum (West, 1976*a*, 1976*b*; Oster-Granite & Gearhart, 1981) – suggest that clones are relatively small, with a mean number of no more than 10 cells in a linear array (i.e. in sections of tissues of one cell thickness), and this has been interpreted as evidence for the existence of considerable cell mingling during development. Mean clone sizes were calculated from the observed mean patch sizes of tissue sections, assuming a random distribution of progenitors and uniform proliferation of clones (West, 1975). The validity of these assumptions was not tested, however, even though appropriate mathematical models are available: size frequency distributions of clones derived through non-differential proliferation of randomly spaced single progenitors will conform to a geometric distribution, while differential growth or mixing, for example, would yield a more skewed distribution (Roach, 1968).

In the present analysis we devised a method for the preparation of mucosal sheets (Schmidt, Wilkinson & Ponder, 1984) which renders the entire population of patches of intestinal crypts in mammalian chimaeras accessible for direct two-dimensional study and statistical analysis. Intestinal crypts of Lieberkühn are three-dimensional structures, forming morphological and functional units of a complex sheet of epithelium (Potten, Chwalinski & Khokhar, 1982). They are suitable for the study of two-dimensional clonal proliferation for the following reasons. (1) After their formation (just after birth in mice) crypts proliferate by fission (Potten *et al.* 1982). (2) In adult chimaeras, crypts are always of a single parental genotype (Ponder *et al.* 1985). In our system, the basic units of a patch are therefore intestinal crypts rather than cells. We estimate that the number of crypts increases from the neonatal period to the adult by at least 20-fold (own unpublished data). (3) The pattern of the mosaic crypt population may be demonstrated by an immunohistochemical method based on a strain-specific difference in expression of binding sites for *Dolichos biflorus* agglutinin (Ponder & Wilkinson, 1983; Schmidt *et al.* 1984).

We determined the large-scale arrangement of the chimaeric components, and established size frequency distributions of patch sizes which were then tested against the two theoretical models for clonal growth (see above). As a representative of a highly skewed distribution we chose the negative binomial distribution which is a contagious distribution derived from the theory of infectious diseases, in which the occurrence of an event is more likely if other events occur in its vicinity (Ross, 1980). It is consequently more skewed than the geometric distribution.

MATERIALS AND METHODS

Mice

Nine DDK↔B6, four RIII↔B6 (Dr M. Wood, MRC Laboratories, Carshalton) and four DDK↔C3H (Dr M. Buehr, MRC Mammalian Development Unit) aggregation chimaeras

(Table 1) were constructed according to methods (Ponder *et al.* 1983) based on those described by Mintz (1971). The chimaeras were between 2 days and 17 weeks old.

Preparation and staining of intestines

Sheets of intestinal mucosa were prepared by dissecting free the entire mucosa from the muscles of the gut wall. The sheets were pinned out flat and fixed overnight in 10 % buffered formol saline (for details see Schmidt *et al.* 1984). The fixed preparation was incubated for 30 min in 0·1 % phenylhydrazine HCl to block endogenous peroxidase, and then for 24 h at room temperature with *Dolichos biflorus* agglutinin–peroxidase conjugate. Between each incubation the preparation was carefully washed in phosphate-buffered saline pH 7·5 (PBS) containing 0·5 % bovine serum albumin. The peroxidase was subsequently demonstrated using 3'3' diaminobenzidine (DAB) (Sigma) as substrate, yielding a brown reaction product.

Analysis

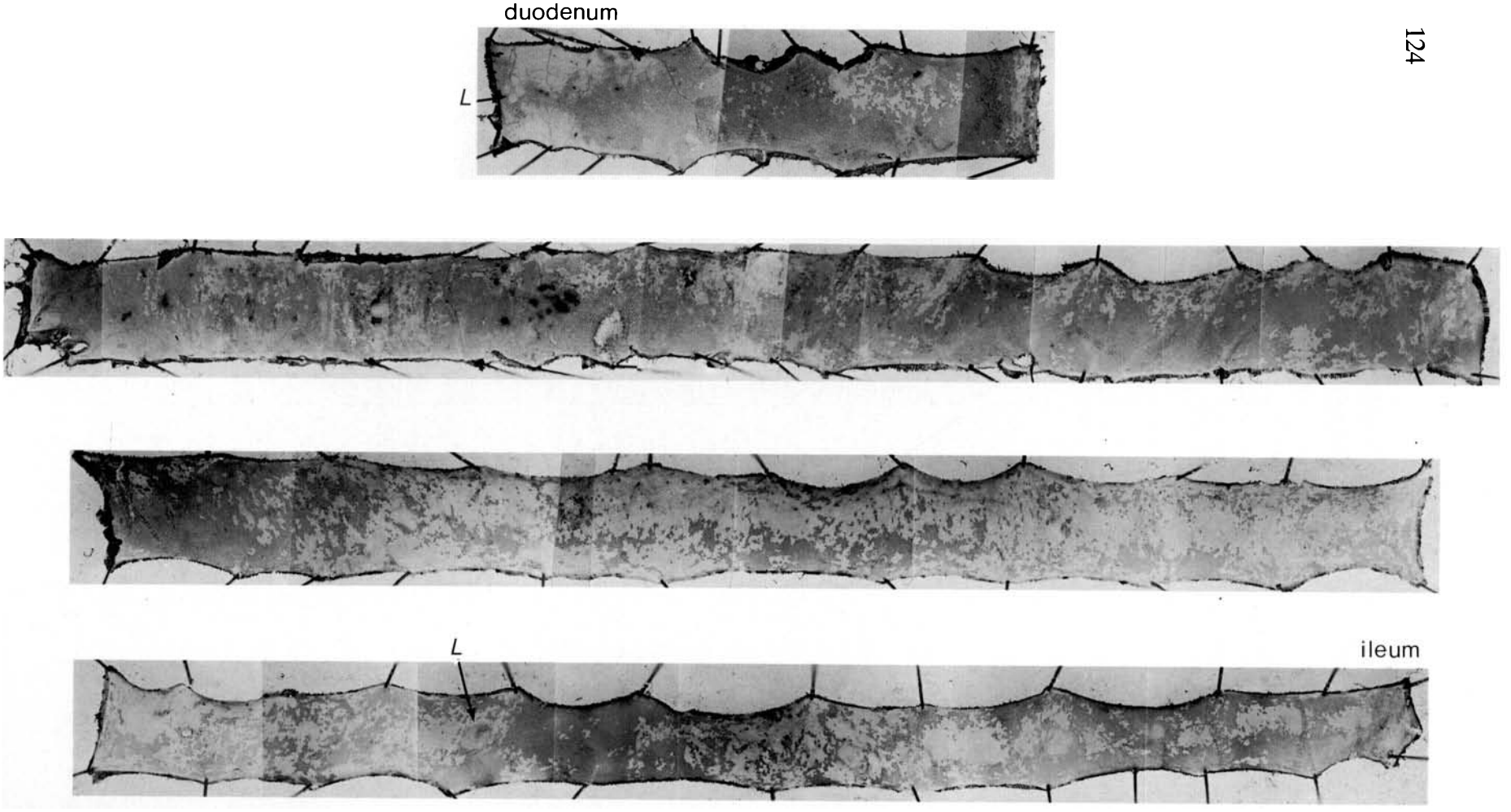
The stained preparations were photographed on Ilford Pan F film (50 ASA), using a Bowens Illumitran adapted to take a Leitz (Wetzlar) macrolens (80 mm; from an optical bench). Photomontages of entire mucosal sheets were made (Fig. 1); from these we determined the relative proportions of the chimaeric components along the length of a small intestine, and recorded the numbers and sizes of discrete patches. Relative proportions were derived from 1029 equally spaced transects (using a MOP Videoplan, Kontron) which had been drawn onto acetate sheets placed on the photomontage. To average out variability, a non-linear smoothing technique (running medians; Ryan *et al.* 1981) was applied to the data. Calculations were carried out on a Nord 500 computer (University of Reading) and plotted with Ghost 80·5 routines.

The number and sizes of patches – expressed as numbers of crypts per patch – were determined for the two intestines with the most highly unbalanced proportions ($p < 0{\cdot}01$). We restricted this analysis to such specimens for the following reason, which is based on the model by Whitten (1978): patches show maximum aggregation and form a network when the proportions (p) of the two components are balanced ($p = 0{\cdot}5$) (Fig. 1), but when unbalanced ($p < 0{\cdot}01$) (Fig. 3) the minority population exists as discrete patches, each patch essentially representing a single coherent clone (extrapolating from Whitten, 1978, Fig. 13: > 98 % of patches of the minority component represent single coherent clones when $p < 0{\cdot}01$). Therefore, the numbers, sizes and contours of patches, and consequently also of coherent clones (Whitten, 1978), are most reliably determined when the proportions of the two crypt populations depart markedly from equality ($p < 0{\cdot}01$). Size-frequency distributions were fitted to the data for patch numbers and sizes, using the method of maximum likelihood (computer facilities as above; Ross, 1980).

Table 1. *Inbred mouse strains used for the construction of aggregation chimaeras and their capacity to bind* Dolichos biflorus *agglutinin (DBA)-peroxidase*

Strain	DBA binding by the epithelium of the small intestine
C57BL/6JLac (B6)	+
C3H/Bi (C3H)	+
RIII/Lac-*ro* (RIII)	–
DDK (DDK)	–

DBA has specificity for terminal non-reducing N-acetyl galactosamine residues (Etzler, 1977).


duodenum
L
L
ileum

RESULTS

In chimaeric intestinal sheets in which the overall proportions were relatively balanced (eleven mice), the majority of either crypt component formed a complex network (Fig. 1). In contrast, in chimaeras with unbalanced proportions (six mice), the minority component occurred as discrete patches which were amenable for quantitative study (Figs 2–4). Patchy staining did not occur in control, non-chimaeric intestines. Analysis of entire mucosal sheets of the six unbalanced

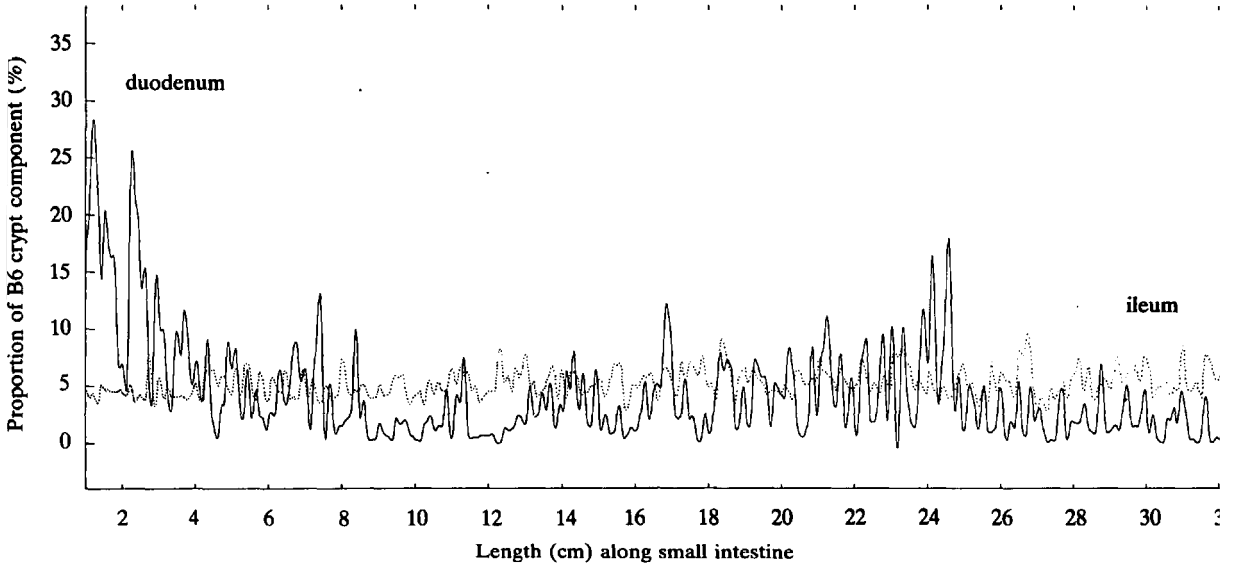


Fig. 2. Non-homogeneous distribution of the B6 crypt component along the length of the small intestine (mucosal sheet) of a 7-week-old DDK↔B6 aggregation chimaera. Overall mean of the proportion of B6 crypts was 4·7 %. Heavy line equals smoothed data (the observed fluctuation partly reflect the patch sizes, c.f. Fig. 4). Dotted line equals simulated smoothed random fluctuations with the same mean as the data. For further explanations see text.

Fig. 1. DDK↔B6 small intestine, 12 weeks old. Photomontage of a complete mucosal sheet (viewed from the abluminal side). Muscles of the gut wall have been removed; for details see Schmidt *et al.* 1985. The sheet was cut into four smaller pieces to facilitate handling of the specimen. DDK crypts do not bind *Dolichos biflorus* agglutinin conjugate and appear as white patches or large areas (contiguous groups of DDK crypts). B6 crypts are stained (dark patches; note that the few very dark spots, predominantly on the second sheet of the intestine, are food showing through from the luminal surface). The highly irregular contours of the mosaic patterns of patches are apparent. Actual length of piece marked 'ileum' (bottom): 11·8 cm. *L*, lymphoid follicle.

chimaeras (three DDK↔B6 ages 17 days, 12 and 17 weeks; three DDK↔C3H ages 7, 8, and 12 weeks) gave the following results.

(1) The relative proportions of the two crypt populations along the length of the small intestine were markedly non-homogeneous (Fig. 2). In each of the six mice the DDK component predominated distally, while the non-DDK component was predominant in the duodenum (although with some local variation).

(2) Individual patches were on the whole relatively small (Fig. 3). Well-defined large patches of crypts with regular outlines were not found. Size frequency distributions of widely separated patches in a juvenile (17 day) and an adult (17 week) (cf Fig. 3) gut with highly unbalanced proportions (the minority type

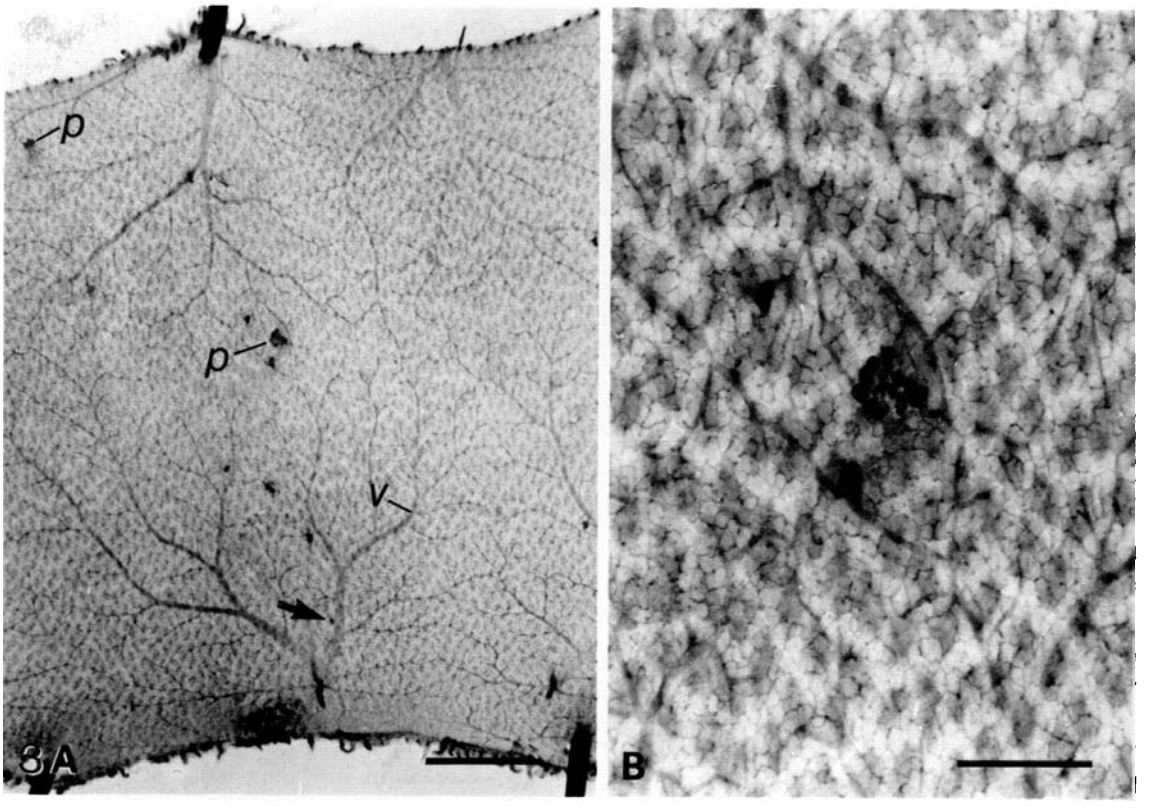


Fig. 3. (A) Patches of crypts (p) in a sheet of intestinal mucosa (Schmidt *et al.* 1984) of a 17-week-old DDK↔B6 aggregation chimaera (viewed from the abluminal side; muscles of the gut wall have been removed). Stained with *Dolichos biflorus* agglutinin–peroxidase. DDK crypts do not stain. The proportion of the B6 crypt component was less than 1% and the patches were therefore discrete. DDK blood vessels (v) bind DBA peroxidase conjugate and are stained. The regular background pattern is formed by the cores of villi on the other side of the mucosal sheet. Arrow indicates isolated B6 crypt. Bar equals 2 mm. (B) Centre part of Fig. 3A at higher magnification showing group of patches. The circular to oval shapes of the densely stained bases (Schmidt *et al.* 1984) of B6 crypts are clearly seen. Bar equals 0·5 mm.

constituting less than 1 %) were highly concave or skewed, because there were a large number of isolated, single crypts and a small number of very much larger patches. The data therefore departed significantly from a geometric distribution ($P < 0{\cdot}05$), but fitted a negative binomial model (Fig. 4).

DISCUSSION

The statistical methods employed in previous analysis of chimaeric tissues (West, 1976*a*, 1976*b*; Oster-Granite & Gearhart, 1981) assumed non-differential proliferation and random arrangement of progenitors and were based on a geometric distribution of clone sizes. Unfortunately, the authors did not test these assumptions; moreover, they consistently gave the mean clone size, which could leave the reader with the incorrect impression that the sizes of clones were distributed normally around the mean. For our data presented in Fig. 4 the mean patch size would be 4·0 crypts, and because the chimaera is highly unbalanced, the mean clone size will be very nearly the same: but this value on its own does not give a clear impression of the actual distribution of patch (or clone) sizes. Our results support Whitten's (1978) view that 'the mean patch size is not a reliable statistic on which to judge mosaics' and that a patch size frequency distribution, carried out for chimaeras with unbalanced genetic components, would be more informative.

The large-scale distribution of patches in unbalanced intestines was markedly non-homogeneous, with relative predominance of the non-DDK component in the duodenum. An anteroposterior gradient was reported for coat pigmentation (West & McLaren, 1976) and for the skull and vertebral column of C57BL/6↔C3H chimaeras (Moore & Mintz, 1972) (this strain combination is not suitable for the methods of the present study, cf Table 1). Such a distribution may reflect a temporal shift of strain-specific selective advantage during development, which proceeds in a cranial to caudal direction (Moore & Mintz, 1972; McLaren, 1976). Qualitative data from other studies of chimaeras (West, 1976*a*; Sanyal & Zielmaker, 1977; Oster-Granite & Gearhart, 1981), and of X-inactivation mosaics (Deol & Whitten, 1972) also indicate that the large-scale distribution of clones is non-uniform. This may therefore occur generally in chimaeric tissues, and also in normal tissue.

Several factors may have contributed to the highly skewed distribution of patch sizes found. (1) Non-homogeneous distribution of patches along the length of the gut on a large scale indicates that clones or their progenitors are probably non-randomly distributed also on a smaller scale. This may have influenced the size frequency distribution, because non-randomness may have led to some aggregation of patches. (2) Theoretical accounts of clonal growth in chimaeras predict that cell migration or mingling will disrupt small clones to a greater extent than large ones (Lewis, 1973). It is conceivable that during development such

mechanisms may result in extremely skewed size frequency distributions of patches, as found in the present study. However, in our system, migration of crypts is highly unlikely as they are an integral structure of the mucosal architecture, so this process could only operate before the time of crypt formation.

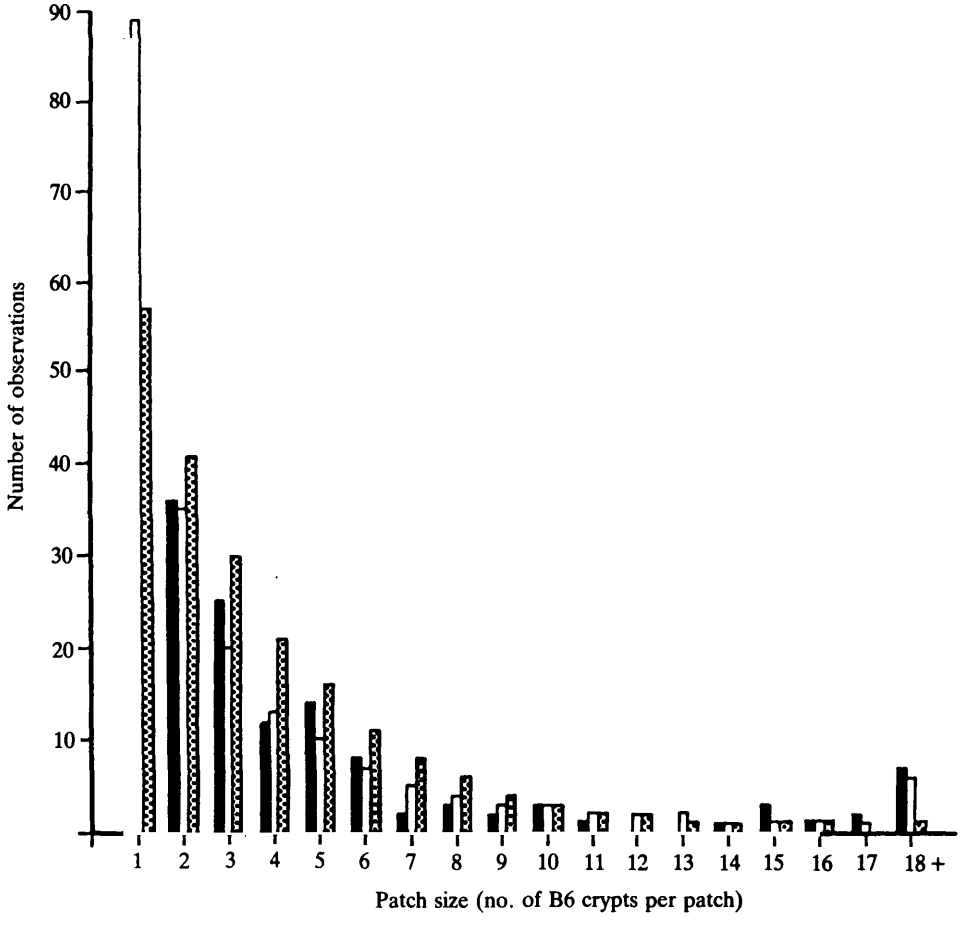


Fig. 4. Size frequency distribution of patches of B6 crypts in the mucosa of the entire small intestine of a DDK↔B6 chimaera (age 17 days). The proportion of the minority crypt population (B6) was less than 1 %. Total number of patches: 207, size range: 1–47 crypts. The observed distribution (solid bars) departed significantly from the fitted Geometric distribution (dotted bars) which would be expected if growth had been by non-differential proliferation of widely dispersed progenitors (significance tested at the 5 % level; this also applies to the second data set, see below). It was highly concave (skewed) and therefore fitted a negative binomial model calculated from the observed data (open bars). Chi-squared of Geometric fit: 64·47 on 16 degrees of freedom (*d.f.*) ($P < 0{\cdot}05$). Negative binomial: 18·73 on 15 *d.f.* Values for a 17-week-old DDK↔B6 chimaera (see text and Fig. 3) were: total number of patches: 1303, size range: 1–134; Geometric fit: 53·01 on 23 *d.f.* ($P < 0{\cdot}05$); negative binomial fit: 32·64 on 22 *d.f.* The intestines of a further four unbalanced chimaeras (see Results) qualitatively substantiate these data.

Furthermore, single crypts or small patches were not concentrated at the boundaries of large patches, which would be expected if they were a consequence of an outward diffusion process ('smearing radius') (Lewis, 1973). Solitary cells have been observed in a variety of chimaeric tissues, and their isolated occurrence was attributed to cell migration (Feder, 1976). Our study shows, however, that such conclusions are not justified without a quantitative analysis: isolated cells which had not proliferated would be expected to occur by chance (see above). (3) Despite the expected chance occurrence of some crypts which have not proliferated, the large numbers of spatially isolated single-crypt patches found, (Figs 3, 4) suggests the possibility that intestinal crypts may proliferate at different rates: while some never or rarely divide others proliferate to a much greater extent. Although it is not proven by our analysis, this interpretation also agrees with the previous suggestion that 'master crypts' with a higher than average proliferation frequency may exist in the intestinal epithelium (Potten *et al.* 1982). (4) The patch size distribution might be affected by unequal proliferation of the two chimaeric components: for example, isolated crypts of one genotype in chimaeric tissue might not proliferate in an environment dominated by the other genotype.

The possible contributions of non-randomness and of differential proliferation, including strain-specific differences, may be further examined by means of computer simulations. Spatial statistics such as the Greig-Smith analysis of variance (Mead, 1974) will also allow us to search for clustering of clones which might indicate their descent from a common progenitor ('descendent clones', West, 1976*a*), and thereby to estimate the number of gut progenitor cells.

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