ORIGINAL ARTICLE

Colonic Cell Proliferation in Normal Mucosa of Patients with Colon Cancer

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Cell kinetics parameters have been analysed in colonic mucosa at different distances from a tumour in patients with colon carcinoma. Total cell number (TCN), ^{3}H thymidine labelling index (TLI), mitotic index (MI), Goblet cell index (GCI) and the distribution of labelled cells along the crypt column (cell position frequency plot) were determined in well-aligned crypts. Total cell number, GCI and the labelled cell position frequency plots were similar in different samples from the same individual. A negative linear correlation between TCN and TLI was observed. The analysis of the cell position plots showed two patterns 1) with a high concentration in the bottom fifth of the crypt and 2) with frequent labelled cells at high positions. Whereas a negative correlation between overall TLI and the percent contribution to the TLI of the lowermost fifth was seen, the correlation was positive for the next 3 fifths and labelling was absent in the last part of the crypt.

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Under steady state conditions in healthy tissue the levels of proliferation are controlled by homeostatic mechanisms which can be influenced by physical, chemical and biological factors to induce new proliferative situations that can be transitory or more permanent if the system is chronically assaulted. In the latter case, a new steady state may be achieved which is characterised by increased mitotic activity and this may be of relevance in carcinogenesis (1–3). Alterations in proliferative and structural aspects of the proliferative compartment are believed to precede cancer development (4). Experimental evidence in rats and mice support this sequence of hyperproliferation, tissue distortion and carcinogenesis (5–8). With this is mind, surgical specimens of human large intestine have been studied on the assumption that although cancer is very specifically located there is the possibility that the whole colonic mucosa may show some pre-neoplastic changes (e.g. increased proliferation) since it may have been exposed to the same chronic stimuli and will have the same genetic constitution. Over the last few years, in vitro (bromodeoxyuridine or tritiated thymidine, ^{3}HTdR) and in vivo (bromodeoxyuridine) techniques have been developed for studying the proliferative activity in tissues taken at surgery (9–15). Because of the potential ethical difficulties associated with bromodeoxyuridine in vivo we have a preference for ^{3}HTdR incubation ex vivo.

The aim of the present study was to evaluate the proliferative activity in the normal mucosa of patients affected by colon cancer. Samples were collected at various distances from the tumour and the ^{3}HTdR labelling index (TLI), the mitotic index (MI) and the Goblet cell index (GCI) were determined. By selecting well-oriented, longitudinally cut crypts the spatial distribution of S-phase cells along the crypt axis can be determined. Analyses were performed on multiple sample from each patient.

MATERIAL AND METHODS

Tissue samples

Samples were obtained at surgery from 13 subjects (9 females, 4 males) with a median age of 73 years (range 56–85 years). The patients all suffered from colonic carcinomas (2 caecal, 1 in the ascending colon, 3 in the transverse colon, 1 in the descending colon and 6 in the sigmoid region of the colon). None of the patients suffered from any other gastrointestinal illness. Immediately after surgery, samples of large intestine mucosa were taken 5 cm

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Table 1

An example of one case of the counting procedure and the variability in total cell number (TCN), thymidine labelling index (TLI), mitotic index (MI) and Goblet cell index (GCI) as mean ± S.E.M.) in the periphery (P), centre (C) and total (T) of the sample taken at 20 cm (A) and 5 cm before (B) and 5 cm after the tumour (C). The total number of sections and crypts analysed are also shown

	Area	No. of sections	No. of crypts	TCN (M ± S.E.)	TLI (M ± S.E.)	MI (M ± S.E.)	GCI (M ± S.E.)
A	T	45	229	58.3 ± 2.4	4.6 ± 0.5	0.03 ± 0.03	7.6 ± 0.7
	P	13	90	60.3 ± 5.5	4.7 ± 0.6	0	6.3 ± 1.2
	C	32	139	57.0 ± 2.7	4.5 ± 0.8	0.04 ± 0.04	8.5 ± 0.5
B	T	51	323	57.8 ± 1.3	5.0 ± 0.7	0.009 ± 0.006	9.2 ± 1.5
	P	23	102	56.7 ± 2.3	5.2 ± 1.4	0.01 ± 0.01	7.3 ± 2.3
	C	28	211	58.7 ± 1.6	4.8 ± 0.9	0.007 ± 0.008	10.7 ± 2.0
C	T	50	341	56.8 ± 1.4	5.7 ± 0.6	0.06 ± 0.02	8.8 ± 0.6
	P	29	244	56.8 ± 2.5	5.7 ± 0.9	0.09 ± 0.01	8.9 ± 0.5
	C	21	97	58.4 ± 0.8	4.6 ± 0.5	0.04 ± 0.003	9.4 ± 1.9

above and below the tumour respectively. Some samples were also taken at 20 cm above (n = 10) or below (n = 3) the tumour.

Tissue treatment

Preliminary studies showed that there were some difficulties in ^{3}HTdR uptake when intact fragments of large intestine were incubated in vitro possibly due to penetration difficulties resulting in only some of the crypts at the periphery of the tissue becoming labelled (11, 16). To overcome this we initially used various concentrations of mucolytic molecules, such as ursodeoxycolic acid and sodium dodecylsulphate for different lengths of time, but such treatments induced morphological alterations in the epithelial cells which may affect permeability and length of the cell cycle. A subsequent approach involved the gentle removal by application of filter paper of the mucus from 2 cm^2 pieces of the mucosa which were then placed on velcro to maintain the orientation of the tissue during sectioning. Each sample was then incubated for 90 min in 9 ml of McCoy's 5A medium (84%), with foetal calf serum (15%), tritiated thymidine (1%, specific activity 740 GBq/mmol, Amersham UK) at 37°C with continuous stirring and a gas environment of 5% CO_2 with air. Before incubation the mucosa was punctured repeatedly in an attempt to increase the probability of ^{3}HTdR uptake. After incubation, the tissue was washed 3 times in cold saline and fixed for 50 min in Carnoy's fixative. Following this, the mucosa was cut into 15–20 fragments of about 0.3–0.4 cm in width that were held separately, recording their position in relation to the original piece of tissue.

After embedding in polystyrene (17) sections were cut at 3 μm and autoradiographs were prepared using Ilford K5 emulsion. The sections were stained with haematoxylin-erythrosin or periodic acid Schiff's (PAS)-haematoxylin.

Scoring techniques

Only crypts that were well aligned longitudinally and which contained at least 1 labelled cell were selected for scoring which was taken as evidence that the ^{3}HTdR had reached the crypt by diffusion. Almost all the crypts in the samples had at least one labelled cell. The total number of epithelial cells, labelled cells, mitotic cells and Goblet cells were counted along the side of the crypt section starting at the crypt base. From these numbers, the thymidine labelling index (TLI), the mitotic index (MI) and the Goblet cell index (GCI) were calculated. Furthermore, the position of the labelled and mitotic cells was recorded along the crypt axis starting with the first cell at the base. Except for one case a minimum of 50 crypt sides were scored in this way; usually many more than 50 were scored. Every 4th section was scored to avoid the possibility of recounting the same crypt. At least 5 fragments for each mucosal sample, at each location, were evaluated for sample-to-sample variation. For the mitotic counting, cells in late prophase, metaphase and anaphase were recorded and for the Goblet cell counting, PAS positive cells with an evident nucleus were recorded. The 13 patients provided 33 samples in total but only in 12 patients could all the scoring be performed resulting in 31 samples for presentation. Mean values ± S.E. were determined and the Wilcoxon rank test was used to test for statistical significance.

The cell positional localisation of labelled cells along the side of a crypt was registered and a frequency plot for a normalised crypt was calculated using a computer programme (18–24).

The data for the frequency plots have been subdivided into 5 crypt compartments each comprising 20% of the crypt length and the relative number of labelled cells in each compartment has been analysed.

RESULTS

There were no morphological mucosal abnormalities in any of the samples. The ^{3}HTdR labelling was uniform in distribution over the peripheral and central tissue specimens (an example of one case is shown in Table 1). This test was repeated in 6 patients and only occasionally were any differences observed. As a consequence of this, we felt

Table 2

Variation in measurements between patients in TCN, TLI, MI and GCI (as mean ± S.E.M.). In the epithelium at different distances before (A), (B) and after (C) the tumour (see Table 1 for details). The results are for 3 patients with cancer in the sigmoid region

	Area	No. of sections	No. of crypts	TCN (M ± S.E.)	TLI (M ± S.E.)	MI (M ± S.E.)	GCI (M ± S.E.)
F 58 yrs	A	28	208	62.4 ± 1.7	5.2 ± 1.5	0.13 ± 0.05	7.2 ± 0.7
	B	24	221	62.8 ± 2.3	5.1 ± 0.5	0.16 ± 0.09	8.0 ± 0.3
	C	24	245	52.6 ± 0.4	6.0 ± 1.4	0.04 ± 0.007	9.2 ± 0.6
M 82 yrs	A	45	158	84.6 ± 2.1	2.2 ± 0.1	0.06 ± 0.03	11.0 ± 0.7
	B	57	209	72.3 ± 2.4	3.1 ± 0.3	0.06 ± 0.04	16.9 ± 0.6
	C	34	104	79.0 ± 3.6	3.0 ± 0.8	0.001 ± 0.001	12.9 ± 2.0
F 62 yrs	A	28	175	52.0 ± 1.2	4.5 ± 0.2	0.30 ± 0.05	1.5 ± 0.1
	B	29	179	56.7 ± 1.8	4.6 ± 1.3	0.18 ± 0.05	5.0 ± 0.3
	C	25	193	62.0 ± 2.5	5.0 ± 0.5	0.05 ± 0.03	4.0 ± 0.7

that counting could be performed in any of the samples chosen. Although this analysis showed that the labelling parameters were the same in each fragment taken from the same sample, or from fragments taken at different positions from the same patient, there were some differences observed when mitotic activity was considered. This is probably due to the fact that mitosis is a short phase in the cell cycle and as a consequence the mitotic index is very low. However, it may also be due to the fact that some mitoses are lost in the interval between resection and fixation. For an example of the sort of variability observed of 3 samples from 3 different patients, see Table 2. Table 3 summarises the results from all 13 patients. The mean value of the number of cells per crypt column (cells along one side of the crypt) was 63.6 (median is 62.4) with a range of 42.8–86.9. No significant differences were observed in any of the cases with distance from the neoplasia.

For the 13 subjects the mean labelling index (TLI) was 5.3% (median 5.0%) with a range of 2.1–10.7%. No specific relationships were seen within this small sample between labelling index and age, sex and site of tumour. There is a statistically significant correlation between TLI and number of cells per crypt column (TCN) as shown by Fig. 1. The TLI is generally greater in those cases with a lower number of epithelial cells per crypt column. In contrast, the number of S-phase cells per crypt (as opposed to TLI) is quite homogeneous with a median value of 3.0 and a mean ± S.E.M. of 3.2 ± 0.2 with a range from 2.0–4.2. The mitotic index varies widely with a range from 0.001–1.24% with a mean value of 0.21% (median 0.13%).

Table 3

Mean ± S.E.M., median and ranges TCN (total cell number), MI (mitotic index) and GCI (goblet cell index) in all 33 samples from the 13 cases

	TCN	TLI%	MI%	GCI%
Mean ± S.E.	63.6 ± 2.1	5.3 ± 0.4	0.21 ± 0.04	5.7 ± 0.7
Median	62.4	5.0	0.13	5.0
Range	41.8–86.9	2.1–10.7	0.001–1.24	1.3–16.9

Amongst all the parameters analysed, this is the only one which, due to the low frequency of mitoses, varies sharply between different crypts in the same section and different mucosal specimens from the same subject.

The percentage of Goblet cells (GCI) in each crypt varies between 1.3 and 16.9% with a median value of 5.0 and a mean of 5.7%. The frequency of Goblet cells in the right colon tends to be lower than in the other regions of the large intestine. In Table 4 the TLI and GCI mean values are presented for three regions of the colon considering the 5 and 20 cm samples together. The GCI values show a significant ($p < 0.05$) increase in the descending colon compared with the ascending region.

The distribution of S-phase cells (labelled cells) along the crypt length is low at the bottom, increases and reaches a maximum generally within the first 20% of the crypt. It decreases higher up the crypt. In most of the crypts, the proliferative compartment is thus confined to the lower half and only exceptionally are labelled cells observed in the uppermost 20% of the crypt. The cell position frequency plot for labelled cells tends to be characteristic for each patient. Typical frequency plots are shown in Fig. 2; Fig. 2a shows the most frequently observed distribution.

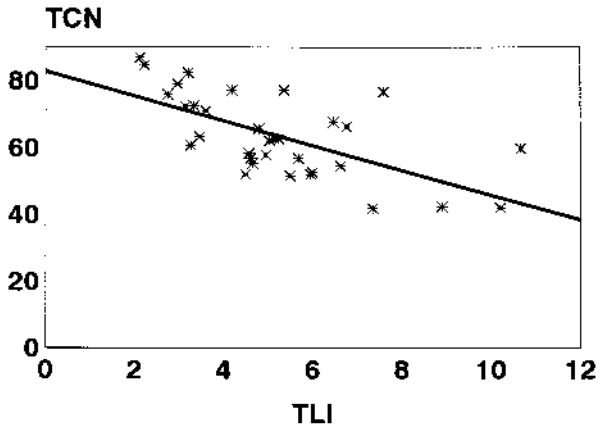

Fig. 1. Relationship between total cell number (TCN) along one side of the crypt and TLI in 33 samples of colonic epithelium ($r = 0.635$, $p = 0.00007$) from 13 patients.

Table 4

*Mean ± S.E.M. and ranges of TLI and GCI in 26 samples of colonic mucosa from 13 cases studied separated into three regions. * statistically significant difference ($p < 0.05$)*

	Ascending	Transverse	Descending
TLI%			
Mean ± S.E.	6.63 ± 0.79	5.39 ± 0.71	4.56 ± 0.50
Range	3.74–10.22	2.22–10.67	3.05–7.35
GCI%			
Mean ± S.E.	2.84 ± 0.71*	5.80 ± 0.97	7.11 ± 1.55*
Range	1.27–7.60	1.47–10.98	2.60–14.92
Number of samples	8	10	8

When the average TLI is high the area under the curve is also high with a tendency to proliferative activity at high cell positions (Fig. 2b). In one subject, a slightly different pattern was noted with a shift in the TLI towards the middle and upper regions of the crypt (Fig. 2c). Generally, the greatest spread in the proliferative compartment was in those subjects with the lowest total number of epithelial cells per crypt column. The present limited data do not show any specific trend in the S-phase cell distribution in different sites of the large bowel, nor any particular trend related to the distance from the colonic tumour.

When the TLI values for each of the 31 samples from the 12 patients are ranked according to increasing TLI values for each of the different fifths of the crypt the lowest fifth generally contains between 50 and 96% of the labelling (see Table 5). The second and third fifths of the crypt usually contain between about 5–25% and 1–5% respectively. In the upper 2 fifths labelled cells do not usually occur. This is the general pattern seen. A second pattern shows less of a difference between the lowest and the immediately adjacent fifth with the percent contribution to the TLI ranging between 7 and 30% in the third fifth with a few labelled cells in the fourth and fifth regions (examples are marked in Table 5).

Linear regression analysis obtained from the 33 samples, showed a negative correlation between the TLI and the percent contribution in the lowest 20% of the crypt ($r = 0.74$) with $p < 0.000001$) (Fig. 3). In contrast, a positive correlation was observed between TLI values and the contribution to the TLI in the second, third and fourth segments ($r = 0.668$; 0.678 and 0.368 respectively with p-values < 0.0001; 0.0001 and 0.04 respectively). No relationship was found between the overall TLI and the percent contribution to the TLI of the uppermost fifth of the crypt.

DISCUSSION

The present study of the total labelling index and labelled cell distribution in the crypt in 'healthy' normal epithelium of colonic cancer patients has been undertaken to investigate possible global changes in proliferation that might predispose to cancer or be part of the processes leading to neoplastic change.

With our technique we see good thymidine diffusion uptake and labelling and did not encounter the problems reported elsewhere (11, 16). Using selected crypts that were

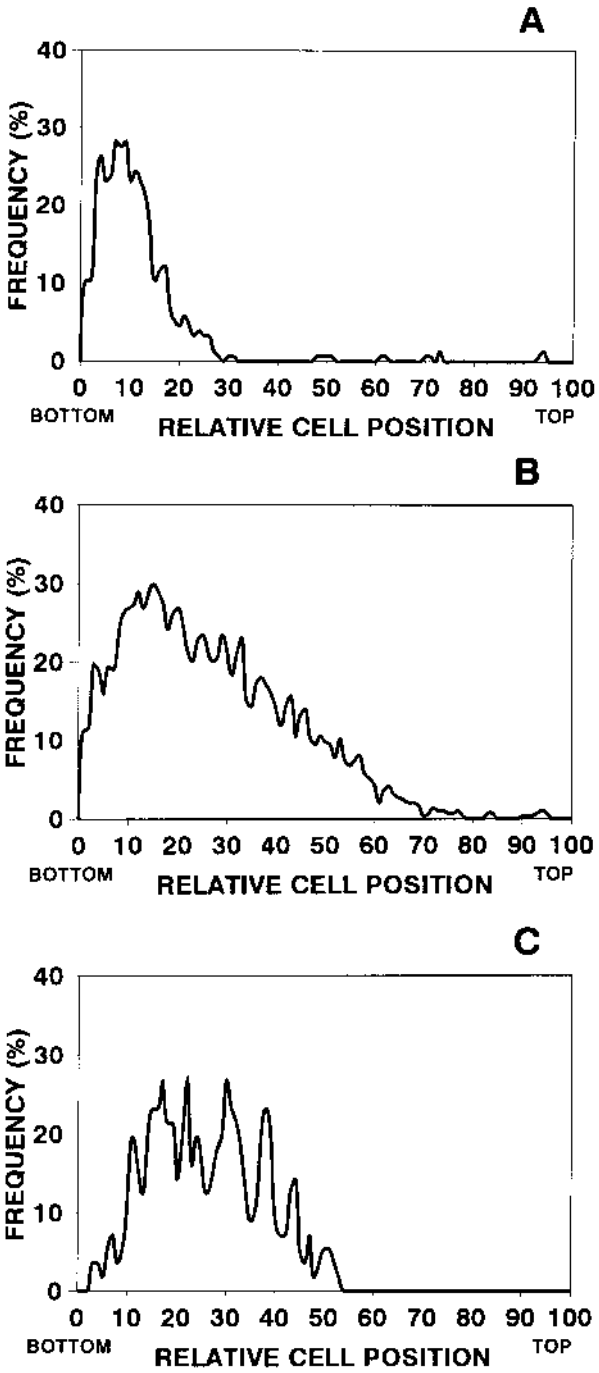

Fig. 2. Individually labelled cell position distributions for three patients showing different types of mucosal proliferation; A) the more frequently observed type of distribution; B) and C) show types of expansion of the proliferative compartment.

Table 5

TLI values for the 31 samples from 12 patients ranked in order. The relative proportion (%) of labelled cells seen in each fifth of the crypts is shown. Samples with possible different patterns are marked

Patient	TLI%	% of labelled cells in each fifth				
		1st	2nd	3rd	4th	5th
1	2.13	88.7	6.6	2.2	2.5	0
2	2.22	88.1	10.2	1.7	0	0
3	2.75	96.3	2.8	0.9	0	0
2	2.97	72.2	22.4	5.4	0	0
2	3.14	67.0	29.7	3.3	0	0
3	3.21	90.3	8.9	0.8	0	0
4	3.26	79.4	18.3	2.2	0	0
5	3.33	92.2	6.2	1.6	0	0
6	3.46	91.1	6.8	0.7	1.0	0.5
6	3.60	88.9	5.3	1.6	1.8	2.4
4	4.18	89.1	9.9	1.0	0	0
7	4.49**	13.6	10.7	48.7	26.2	0.9
8	4.57	58.3	32.3	7.2	2.0	0.2
7	4.60	61.8	25.9	10.2	2.1	0
9	4.65*	54.9	26.8	16.3	2.0	0
10	4.79	68.5	22.4	8.3	0.8	0
8	4.95*	50.3	34.0	13.3	2.4	0
7	5.02	52.1	42.5	3.2	2.1	0
11	5.12	79.7	16.9	2.9	0.5	0
11	5.24	71.0	25.9	3.0	0.2	0
1	5.36*	55.2	32.6	10.6	1.4	0
12	5.49**	24.7	38.0	26.0	11.3	0
3	5.68	49.5	42.5	7.3	0.7	0
3	5.95*	50.0	31.1	13.9	5.1	0
11	5.99*	55.7	28.4	15.1	0.9	0
3	6.47	59.6	32.8	7.2	0.3	0
5	6.63*	35.7	52.6	11.6	0	0
10	6.77*	56.4	28.3	13.5	1.9	0
12	7.35**	25.9	29.6	30.2	12.3	2.0
1	7.59	54.1	31.3	12.3	2.1	0
6	10.67*	42.9	36.2	17.7	2.8	0.4

* High LI% in 2nd fifth of the crypt.
** High LI% in 3rd fifth of crypt.

longitudinally aligned, we have been able to measure proliferation in relation to cell position in the crypt. Because relatively few subjects were analysed, no conclusions could be drawn concerning the effects on proliferation of age, sex, site or stage of colonic cancer, nor between position in the gut and distance from the tumour. However, the present data would seem to suggest that none of these factors have a large effect. The frequency plots for labelled cells along a side of the crypt reported here after incubation with ^{3}HTdR are similar in general shape to those obtained from 77 English patients injected with bromodeoxyuridine (BrdUrd) (14, 15) (see Fig. 4) which confirms the validity of the in vitro method. The general pattern is similar but in the earlier study more labelled cells were seen in the second fifth of the crypts. The differences may be explained by the different methods of labelling. The in vivo administration of BrdUrd gives a physiological

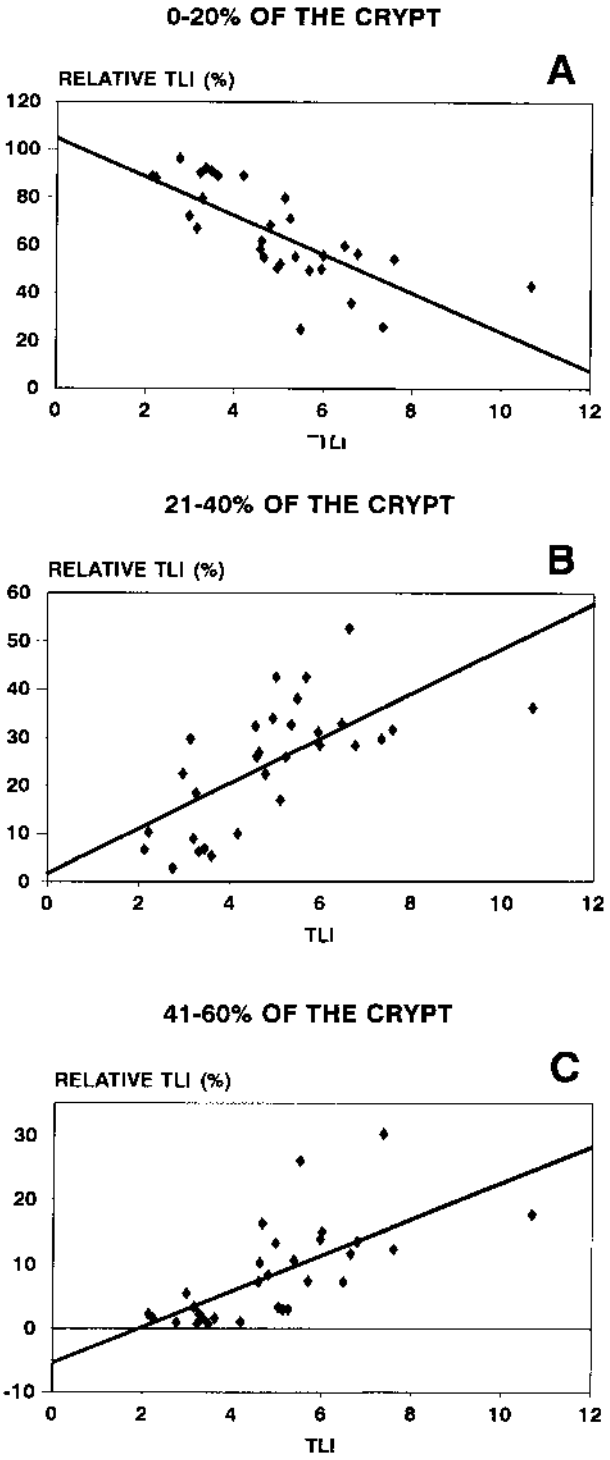

Fig. 3. Linear regression analysis of the relative percent contribution (relative TLI) to the total thymidine labelling index (TLI) observed in the various compartments (fifths) of the crypt: A) lowest fifth (0–20%) $r = 0.740$, $p = 10^{-6}$; B) the second fifth (21–40%), $r = 0.688$ $p = 0.00006$; C) the third fifth (41–60%), $r = 0.678$ $p = 0.00004$.

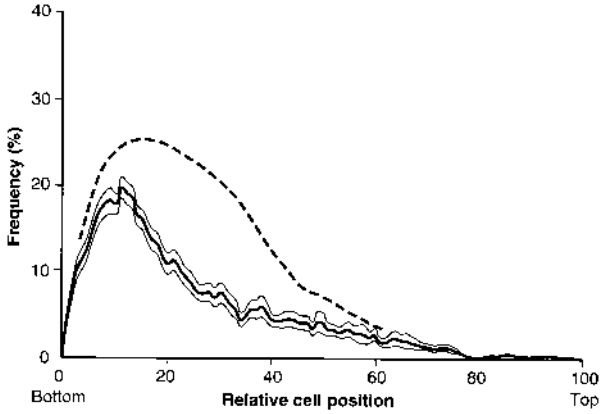

Fig. 4. Labelled cell distribution in 77 samples of human colonic mucosa after in vivo administration of BrUdR presented elsewhere (14, 15) compared with the overall average labelled cell distribution with its standard error limits for the 33 samples of human colonic epithelium from 13 colonic carcinoma patients analysed here.

availability of label rather than a reliance on diffusion. There is also the possibility that some differences may be attributable to the differences in life-style between the two groups of patients (Italian-v-English).

The fact that the total number of cells per crypts column in different samples from the same subject did not vary suggests that the size of the crypt may be typical of an individual and likely to be largely constant throughout the area of the large bowel examined. The mitotic index is not a particularly useful cell proliferation parameter because of the small numbers of cells in mitosis. The total number of labelled cells does not vary much but there is some variability in the labelling index because of differences in cell numbers per crypt. A negative linear correlation between the TLI and the total cell number was observed.

From the frequency cell plots for labelled cells it is evident that the majority of labelled cells are within the lowest fifth of the crypt with a steep decline in the number of labelled cells in the next fifth and an absence of label in the upper fifth. These frequency plots seem to be typical for each individual and even to be similar in all the specimens from different sites from a particular individual. Only occasionally did one of the specimens in a series from an individual differ from the others. If this was the case, then the differences were either of 1 or 2 types.

Firstly, a high number of labelled cells more or less equally distributed between the 2 lowermost fifths with a general decline thereafter. The results show that the increased proliferative activity in some crypts does not correspond to an increased labelled cell frequency in the lower regions of the crypt, but in contrast, an upward shift of the zone of proliferation as was observed elsewhere for the proliferative changes in polyposis coli (14). Secondly, in just a few cases there was an increase in the distribution of labelled cells at higher positions without a corresponding increase in the overall TLI.

The frequency distributions of labelled cells were not affected by the site of the tumour or the proximity of the specimen to the tumour. Generally, high TLI values corresponded to lower Goblet cell frequencies and lower Goblet cell indices corresponded to a greater spread of the proliferative compartment which confirms somewhat, the hypothesis that the number of Goblet cells and hence differentiation is related inversely to the level of proliferation.

Earlier studies in animals exposed to abdominal sublethal doses of ionising radiation showed that during the repopulation phase in the small intestine, there was a significant increase in the number of S-phase and mitotic cells (18–20, 25–29). These changes are co-ordinated with an expansion of the proliferative compartment and labelled cells can be found in the upper parts of the crypts where, under normal conditions, the cells would be differentiated and characterised by brush border enzyme synthesis. At these times, Goblet cells are infrequent. These changes are transitory and disappear some days after irradiation, later the higher the dose of radiation. After the repopulation phase, when the principal columnar cell line prevails, the proliferative compartment returns to normal dimensions with the normal production of markers of differentiation such as Goblet cells and brush border enzymes (20, 21, 24, 30–33).

The results of the present study, although based on relatively few patients and in a restricted area of the large intestine, show no sign of the general changes in proliferative activity associated with cancer which have been considered by some as an indicator of high risk for neoplasia (6). In the present series, the variations seen in the total cell number and the TLI are linked more closely with variability between individual patients. Only occasional modifications were present, and these seemed to be unrelated to site or position in relation to the tumour.

In summary, the present study has shown or confirmed some interesting aspects of colonic mucosal biology. Firstly, there is an increasing trend in Goblet cell number from the ascending to the sigmoid colon. Secondly, the total cell number, i.e. crypt size in the area examined, seems to be a characteristic for an individual patient. Thirdly, the number of S-phase (or labelled) cells is remarkably constant in spite of variations in the total cell number per crypt, and lastly, a spread in the proliferative compartment towards the surface of the mucosa corresponds generally with an increase in TLI. This is not a consistent finding since sometimes tumours can be found in patients with a colonic mucosa which characteristically has a low proliferative activity. This observation is in contrast with some previous reports where there was an association between the presence of tumour and an evident general increase in proliferative activity (34). However, other recent studies have suggested that the proliferative activity in colonic mucosa adjacent to tumours is generally

depressed when compared with sites distant from the tumour (14, 15).

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