# Quantitative Analysis of Cell Population in Mouse Intestinal Epithelium Using Citric Acid (37228)

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The concept of continuous and rapid renewal of the epithelium of the normal intestine has been established in recent years by cell kinetics analysis using the method of $^3$H-thymidine autoradiography (1–4), but little is known about quantitative analysis of population with proliferative and differentiation cells among epithelial cells. Recently Hagemann *et al.* (5, 6) devised a method for estimating the number of epithelial cells in a small intestine by measuring the radioactivity of $^3$H-thymidine in the intestinal epithelium and by applying the squash method for villus and crypt.

In the studies reported below, the number of epithelial cells in a small intestine was calculated by using a number of such cells prepared with citric acid (7, 8) and through the characterization of the cell nucleus with histological observation.

*Materials and Methods. Isolation of cell nuclei.* The small intestine of 8–10-week-old female mice (ICR/JCL) was removed, from the pyloric sphincter to the ileocecal junction, immediately after sacrifice by cervical dislocation. Procedure for cell separation was performed at 4°; blood vessels and fat in the mesentery were removed, and the intestine was then slit longitudinally and rinsed thoroughly in ice-cold Hank's solution. After being blotted and weighed, the intestine was then put into a Petri dish containing 2 ml of 1% ice-cold citric acid solution and minced with scissors into approximately 2-mm square segments. All segments were then mixed at 1–2 $\times$ $10^4$ rpm for 5 min in a Waring Blendor or like mixer and suspended in 20–25 ml of 1% ice-cold citric acid so as to obtain a tissue homogenate. One drop of octyl alcohol was added to the resulting homogenate to reduce foam, after which it was rehomogenized at low speed for about 5 sec. The homogenate obtained was centrifuged in a swing rotor at 500*g* for 10 min. The supernatant was discarded, and the sediment defined as a nucleus fraction was resuspended in 15–20 ml of 0.2% citric acid. A schema of this technique is shown in Table I.

*Estimation of cell number.* Cells were calculated both by the differential count of isolated cell nuclei and by histological observation. In the isolation of the cell nuclei, the percentage recovered was estimated by determining DNA content in the nucleus and supernatant fraction. DNA content was determined by Burton's method (9). The numbers of round- and oval-shaped cell nuclei were counted under the phase-contrast microscope, but fibroblastic cell nuclei were carefully excluded. Four histological sites were selected in the intestine: (site 1) the duodenum immediately adjacent to the pylorus; (site 2) the jejunum, one-third of the distance from the duodenum; (site 3) the ileum, two-thirds of the distance from the duodenum; and (site 4) the ileum immediately adjacent to the ileocecal junction.

A 2-cm-length segment was removed from each site, fixed with Bouin's solution, embedded in paraffin, and sectioned at 5-$\mu$m thickness. The sections were then stained with Mayer's hematoxylin and eosin, and the major and minor nuclear axes of each cell were measured in histological section under a micrometer-equipped microscope. The number of each cell type per traversed section was counted in four parts of the section. Cells were classified into six types according to nuclear shapes; epithelial (crypt,

TABLE I. Procedure for Isolation of Cell Nucleus.

| |
|---|
| Dissecting small intestine after washing with Hank's solution |
| ↓ |
| Homogenization in 1% citric acid (Waring Blendor, 10 min) |
| ↓ |
| Centrifugation, 500g, 5 min (supernatant discarded) |
| ↓ |
| Resuspending nucleus fraction in 0.2% citric acid |
| ↓ |
| Counting nuclei with hemocytometer and smearing for autoradiography |

villus), submucosal, muscle, serosal, and lymphoid cells, respectively.

The number of epithelial cells labeled with tritiated thymidine ($^3$H-TdR, 10 Ci/mmole) was obtained in the following way. Each mouse was injected ip with 1 $\mu$Ci of $^3$H-TdR per gram body weight, and a suspension of cell nuclei was prepared 1 hr after the injection. The cell nuclei were smeared on a glass slide, dipped in Sakura NR-M2 emulsion for autoradiography, and exposed for 2 weeks; after developing, it was stained with Mayer's hematoxylin. The labeling index of the epithelial cells was estimated by counting the number of labeled round- and oval-shaped nuclei. The total number of labeled cells was calculated from the total number of epithelial cells and the labeling index.

*Results. Morphology of isolated nuclei.* The isolated nuclei of an intestinal cell observed under the phase-contrast microscope are shown in Fig. 1.

Very slender shaped nuclei were regarded as muscle cell nuclei, and those with oval, slightly-elongated, large configuration were considered as epithelial cell nuclei. The nucleus shape with each cell type was classified with histological observation of the small intestine.

*Recovery rate of isolated nuclei by DNA determination.* The DNA content of the cytoplasmic and nuclear fraction is shown in Table II. The recovery of the DNA content of the nuclear fraction was found to be more than 90%.

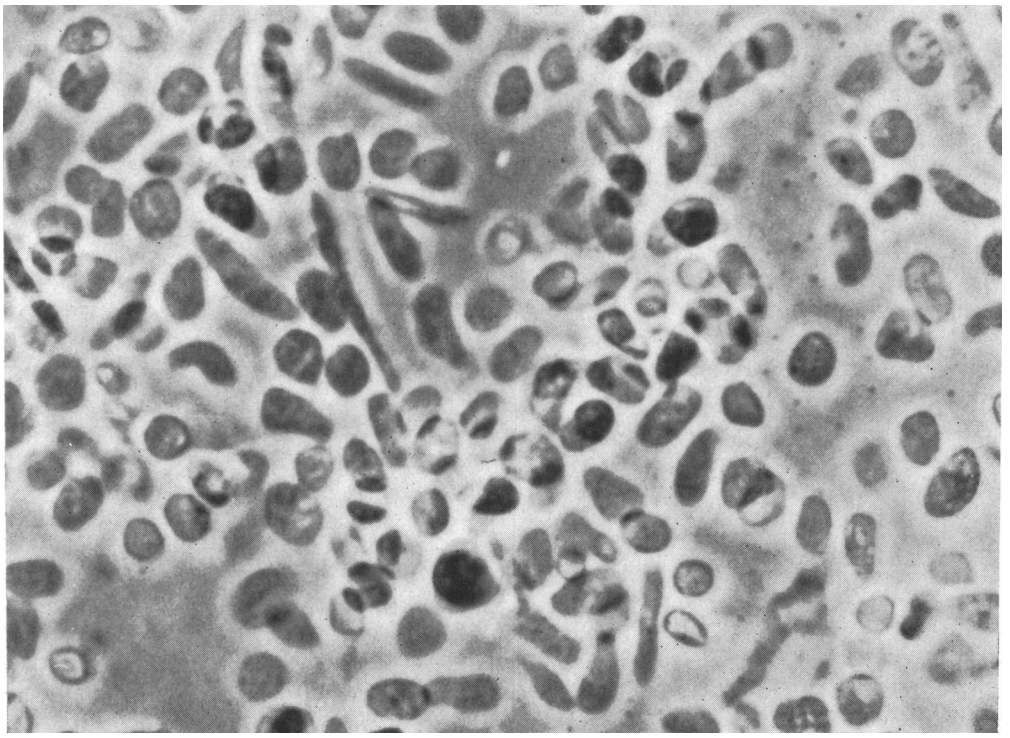

FIG. 1. Phase contrast micrograph of isolated cell nuclei (×900).

TABLE II. DNA Content of Cytoplasmic (Fraction I) and Nuclear Fraction (Fraction II) in Small Intestine.

| | DNA content ($Y$) | Recovery ratio of nuclear DNA |
|---|---|---|
| Fraction I | $750 \pm 347$[a] | |
| Fraction II | $10355 \pm 1596$ | |
| Fraction II / Fraction I + Fraction II | | $0.928 \pm 0.041$ |

[a] Values represent mean $\pm$ standard deviation with five animals.

*Size distribution of nuclei in each cell type.* The size distribution of nuclei obtained by histological observation is shown in Fig. 2. Nucleus size was measured with at least 300 cells of each cell type. The mean value and standard deviation of major and minor nuclear axis in each cell type are shown in Table III. Epithelial cell nuclei could be distinguished from other cell-type nuclei such as the nuclei of submucosal, muscle, lymphoid, and serosal cells as shown in Fig. 2 and Table III.

*Number of cells per section.* The number of cells per type observed in the whole field of a traversed section is shown in Table IV. The proportion of epithelial cells in the population of submucosal and epithelial cells was calculated from the results in Table IV and represented in Table V.

The shapes of muscle or serosal cell nuclei were easily distinguishable from those of epithelial cell nuclei. In addition, the proportion of muscle and serosal cells to epithelial cells was very small, as shown in the last column of Table IV. The number of muscle and serosal cells therefore might be negligible in the estimation of the epithelial cell number even if these cells were mistakenly counted among epithelial cell nuclei. Since a submucosal cell has a fibroblastic- and irregular-shaped nucleus, and its size-distribution is markedly differed from that of epithelial cell nuclei (Table III), the two types of nuclei were generally distinguishable. But oval-shaped epithelial-cell nuclei were not invariably distinguishable from irregular-shaped submucosal-cell nuclei because the size-distribution of the former partially coincided with that of the latter.

The proportion of submucosal cells relative to the total number of submucosal and epithelial cells was estimated as 16%, and the fibroblastic-shaped nuclei were easily distinguishable. Thus the total epithelial cell estimate would seem to represent more than 84% of the actual epithelial cell population. Consequently, the number of epithelial cells could be estimated by counting both the oval- and round-shaped nuclei in the iso-

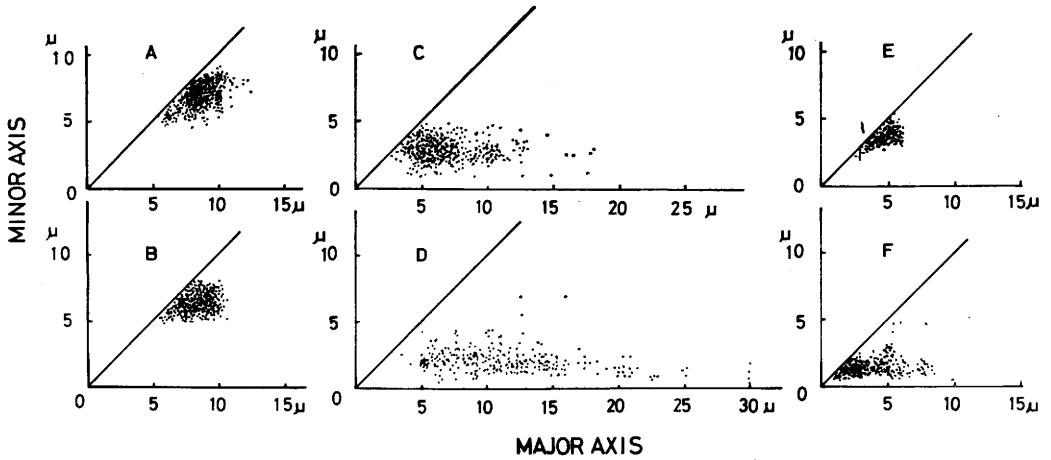


FIG. 2. Distribution of cells as to nuclear size in the microscopic section of intestine. Abscissa, major axis ($\mu$m); ordinate, minor axis ($\mu$m); A, crypt epithelial cell nucleus; B, villus epithelial cell nucleus; C, submucosal cell nucleus; D, muscle cell nucleus; E, lymphoid cell nucleus; F, serosal cell nucleus.

TABLE IV. Comparative Number of Cells Per Type Per Section at Four Small Intestine Sites.

| Site[a] | VE[b] | VS | CE | CS | VE + CE | VS + CS | Serosa | Muscle |
|---|---|---|---|---|---|---|---|---|
| 1 | 15617 | 3538 | 12230 | 2050 | 27848 | 5589 | 924 | 956 |
| 2 | 18102 | 3923 | 12884 | 1913 | 30986 | 5835 | 1056 | 785 |
| 3 | 17776 | 4297 | 15614 | 2348 | 33390 | 6643 | 1117 | 1275 |
| 4 | 14331 | 3318 | 13841 | 2217 | 28170 | 5536 | 1201 | 882 |
| Mean | 16736 ± 1706 | 3846 ± 403 | 14112 ± 1731 | 2159 ± 182 | 30848 ± 2133 | 5901 ± 443 | 1124 ± 59 | 980 ± 212 |

[a] Site 1, duodenum; site 2, jejunum; site 3, ileum; site 4, ileum, adjacent to ileoccal junction.
[b] Abbreviations used: VE, villus epithelial cell; CE, crypt epithelial cell; VS, villus submucosal cell; CS, crypt submucosal cell.

TABLE III. The Distribution of Cell Nuclei as to Size.

| Cell nucleus | Major axis ($\mu$m)[a] | Minor axis ($\mu$m)[a] |
|---|---|---|
| Epithelial cell (crypt) | 8.56 ± 4.44 | 6.74 ± 1.56 |
| Epithelial cell (villus) | 8.53 ± 1.16 | 6.13 ± 0.82 |
| Submucosal cell | 7.59 ± 2.62 | 2.89 ± 1.02 |
| Lymphoid cell | 4.52 ± 0.88 | 3.64 ± 0.63 |
| Serosal cell | 3.71 ± 1.85 | 1.37 ± 0.67 |
| Muscle cell | 11.55 ± 5.32 | 2.01 ± 0.90 |

[a] Under a microscope equipped with a micrometer, the major and minor nuclear axis of each cell was measured in histological section of the intestine.

lated nuclear suspension under the phase-contrast microscope. The total number of epithelial cells in the small intestine was $5.9 \pm 0.5 \times 10^8$, and that of the labeled cell nuclei was $7.6 \pm 1.5 \times 10^7$ with nuclear autoradiography. From the total number of epithelial cells in the small intestine and the ratio of epithelial cell number to that of villus and crypt epithelial cells observed in the traversed section (Table V), the total number of epithelial cells in the crypt and villus was calculated to be $2.7 \pm 0.2 \times 10^8$ and $3.2 \pm 0.3 \times 10^8$, respectively. The number of proliferative cells in the crypt cell population, calculated from the durations of DNA synthesizing phase and cell cycle (7.7, 14.5 hr), and the estimated number of labeled cells, was $1.43 \times 10^8$ cells in the small intestine.

*Discussion.* The reliability of the method reported here depends on the recognition of the difference in size and shape between the isolated nuclear types. In an isolated nuclear morphology, the epithelial cell nuclei were easily distinguished from fibloblastic cell nuclei, but the difference in shape between epithelial cell nuclei and the irregular-shaped nuclei of the submucosal cell could not always be so readily recognized. However, the proportion of irregular-shaped cell nuclei in the submucosal cell population may well be less than 16% of the total number of epithelial and submucosal cells (Fig. 2, Table V). Therefore it was assumed that the epithelial cell number arrived at in this report covered approximately 90% of the epithelial cells in the small intestine. Besides the cer-

TABLE V. The Ratio of Epithelial Cell Number to Submucosal Cell Number in Both the Villus and Crypt.

| Site | VE[a] / VE + CE | CE / VE + CE | VE / VE + VS | CE / CE + CS | TE / TE + TS |
|---|---|---|---|---|---|
| 1 | 0.560 | 0.440 | 0.815 | 0.856 | 0.833 |
| 2 | 0.584 | 0.416 | 0.822 | 0.871 | 0.842 |
| 3 | 0.532 | 0.468 | 0.805 | 0.869 | 0.834 |
| 4 | 0.509 | 0.491 | 0.812 | 0.862 | 0.836 |
| Mean | 0.546 | 0.458 | 0.813 | 0.867 | 0.837 |
| ± SD | ±0.028 | ±0.031 | ±0.007 | ±0.004 | ±0.003 |

[a] Abbreviations used: TE, total epithelial cells; TS, total submucosal cells; SD, standard deviation.

tain number of the submucosal cells counted as epithelial cells, the cells of Brunner's gland in the duodenum were also probably so included because the shape of the cell nuclei in said gland is the same as that of epithelial cell nuclei. Still, Brunner's gland is relatively small in proportion to the total small intestine, and its cell population may well be negligible in the overall estimation of the number of epithelial cells.

Hagemann *et al.* (5) estimated that the total number of intestinal epithelial cells in the C57BL/6 mouse was $5.6 \times 10^8$; proliferative cells, $1.8 \times 10^8$; and DNA-synthesizing cells (S cell), $1.0 \times 10^8$, respectively. In spite of difference in strain and sex, the total number of intestinal epithelial cells in the present results is remarkably similar to the above authors' data except that the number of S cells is slightly lower. This discrepancy might be ascribed to the following two causes; (1) the number of labeled cell nuclei reported here was obtained by counting only round- and oval-shaped cell nuclei, and (2) the cell number estimate of Hagemann *et al.* rests on the premise that the proliferative cell alone belongs to the epithelial cell and that the presence of proliferative cells of muscle and lymphoid type can be set aside.

The present method may be characterized as a simpler procedure for calculating epithelial cells over against the usual histological method. It can be applied to analyze cell population kinetics following X-irradiation or chemical treatment. The method of Hagemann *et al.* has been used on the assumption that the radioactivity of $^3$H-TdR incorporated into DNA in a given time of the DNA-synthesizing phase is constant; thus, it is not adequate to analyze cell population following X-irradiation because it is well known that radioactivity is often variable after treatment (10–12). The authors' method is applicable to other tissues, for example, liver or various lymphoid tissues, if the recovery percentage of the estimated cell number can be obtained.

*Summary.* The total epithelial cells in small intestine of female ICR/JCL mice was $5.9 \pm 0.5 \times 10^8$ as calculated from the number of oval- and round-shaped cell nuclei isolated by citric acid; DNA-synthesizing cells were estimated at $7.6 \pm 1.5 \times 10^7$ by autoradiography. This appears to be a simpler, more accurate method of totaling epithelial cells than any such heretofore.

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