Jpn. J. Cancer Res. 80, 637–642, July 1989

Random Segregation of DNA Strands in Epidermal Basal Cells

Toshio Kuroki and Yoshinori Murakami[1]

Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108

According to the hypothesis proposed by Cairns, stem cells retain the older of the two parental DNA strands, whereas differentiating daughter cells receive the newly synthesized strand, so that a set of "immortal strands" persists in stem cells through successive cell divisions. To test this hypothesis, five successive divisions were induced in basal epidermal cells *in vivo* by two injections of cholera toxin into mouse skin and cells labeled with [^{3}H]thymidine at the first cell cycle were chased for 50 days. If selective segregation occurs, the labeled strand should be transferred into a non-stem daughter cell after the second division and labeled cells would eventually be eliminated from the epidermis. However, the results suggest random segregation of DNA strands in epidermal basal cells. Labeled basal cells were persistently present throughout the whole epidermis for 50 days. Furthermore, labeled mitotic cells were found after the third division and their numbers of grains decreased exponentially through 5 cycles of divisions.

Key words: Immortal DNA strand hypothesis — Stem cells — Epidermal basal cells — Cholera toxin

Stem cells are defined as cell types capable of extensive self-maintenance throughout the whole life span of organisms.[1)] Upon division, stem cells produce one daughter cell which remains as a stem cell and one daughter cell which is committed to differentiation. For possible protection of stem cells from genetic damage, Cairns[2)] proposed a hypothetical model that stem cells retain the older of the two parental DNA strands, whereas differentiating daughter cells receive the newly synthesized strand, so that a set of "immortal strands" persists in stem cells through successive cell divisions. Stem cells could be protected in this manner against errors arising during DNA replication.

Because the concept of stem cells is established as a mechanism for homeostasis of tissue structure and cell differentiation, this hypothesis can only be verified *in vivo*. When cultured *in vitro*, cells are free from the structure of tissues and hierarchy of cell populations, and thus in principle no stem cells exist in cultures. The location of stem cells has been identified only in a few tissues such as crypts of the small and large intestines, filiform papilla of the tongue and basal layer of the epidermis (for a monograph, see Ref. 3). However even in these tissues, the exact identification of the stem cells is uncertain.

We reported previously that a single injection of cholera toxin into the back of mice induces two successive synchronous divisions of basal cells in the epidermis *in vivo*, showing peaks of DNA synthesis and mitosis at about 24 and 48 h after the injection.[4)] These two successive divisions are achieved by division of the same cells twice, not by that of different cells at the first and second peaks.[5)] Stimulation of cell divisions caused epidermal hyperplasia which reached maximum on day 4 and returned to the normal thickness on day 7. We found that further peaks of mitosis were induced by a 2nd injection of cholera toxin on day 4.

We examined the immortal strand hypothesis by labeling of epidermal basal cells with [^{3}H]thymidine during the first cycle of five successive divisions which were induced by injections of cholera toxin into mouse skin *in vivo*. If the hypothesis is correct, the labeled strand should be transferred into a non-stem daughter cell after the second division and be eventually eliminated. We found, however, that labeled basal cells were persistently present throughout the whole layer of the epidermis during the observation period of 50 days. Furthermore, mitotic basal cells were labeled persistently during this period and their numbers of grains decreased exponentially through cell divisions. These data cast doubt on the immortal strand hypothesis.

MATERIALS AND METHODS

Chemicals Cholera toxin was purchased from the Chemo-Sero-Therapeutic Research Inst. (Kumamoto). A solution of the toxin was prepared as reported elsewhere.[4)] [*methyl*-^{3}H]Thymidine (43 Ci/mmol) was obtained from Amersham International (Bucks, UK).

Animals and animal experiments Female Sencar mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu). DDD mice were obtained from the breeding house of this institute.

[1] Present address: Oncogene Division, The National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo 104.

The dorsal skin of mice was shaved with electric clippers 2 days before experiments. Cholera toxin at a dose of 50 ng was injected subcutaneously into the dorsal skin of mice using tongue forceps (ring-shaped, 20 mm in internal diameter) under anesthesia. The forceps were released after 1 h. The second injection of the toxin was done on day 4 at the same site. [^{3}H]Thymidine at a dose of 10 μCi per mouse was injected intraperitoneally twice at 18 and 22 h after the first injection of the toxin, so that the epidermal cells were labeled at the first cycle of DNA synthesis. Mice were killed every 6–10 h during the first 7 days, every 1–2 days on day 8–16 and thereafter once every 1–2 weeks up to day 50. Two mice were used at each point. To avoid the complication of possible circadian rhythm, animals were killed at 6 pm. Demecolcin was injected intraperitoneally at a dose of 50 μg/mouse 3 h before killing to arrest mitotic cells.

Experiments were repeated twice using Sencar and DDD mice. Because essentially the same results were obtained, data with Sencar mice are shown here.

Autoradiography Skin at the site of the injection was fixed with formalin, embedded in paraffin and sectioned. Deparaffinized sections were washed three times with 5% perchloric acid at 4°C to minimize background grains and subjected to autoradiography using NR-M2 emulsion (Konishiroku Photo Co. Ltd., Tokyo). Exposure for 3 months was required for autoradiography, because of the use of a low dose of [^{3}H]thymidine to minimize possible induction of sister chromatid exchanges (SCE). To evaluate labeled basal cells and the mitotic index, more than 500 cells were scored in randomly selected fields. Taking account of the exponential decrease of grains through cell divisions (see Fig. 4), cells with 5 grains were regarded as labeled during the first 9 days, whereas those with 3 grains between day 10–19 and those with a single grain thereafter were counted as labeled cells. Background numbers of grains were negligible: only 2 grains were counted in an area corresponding to 1,000 nuclei.

RESULTS

Strategy The immortal strand hypothesis could be examined by labeling of DNA in stem cells and chasing them for more than three cycles of cell division (Fig. 1).

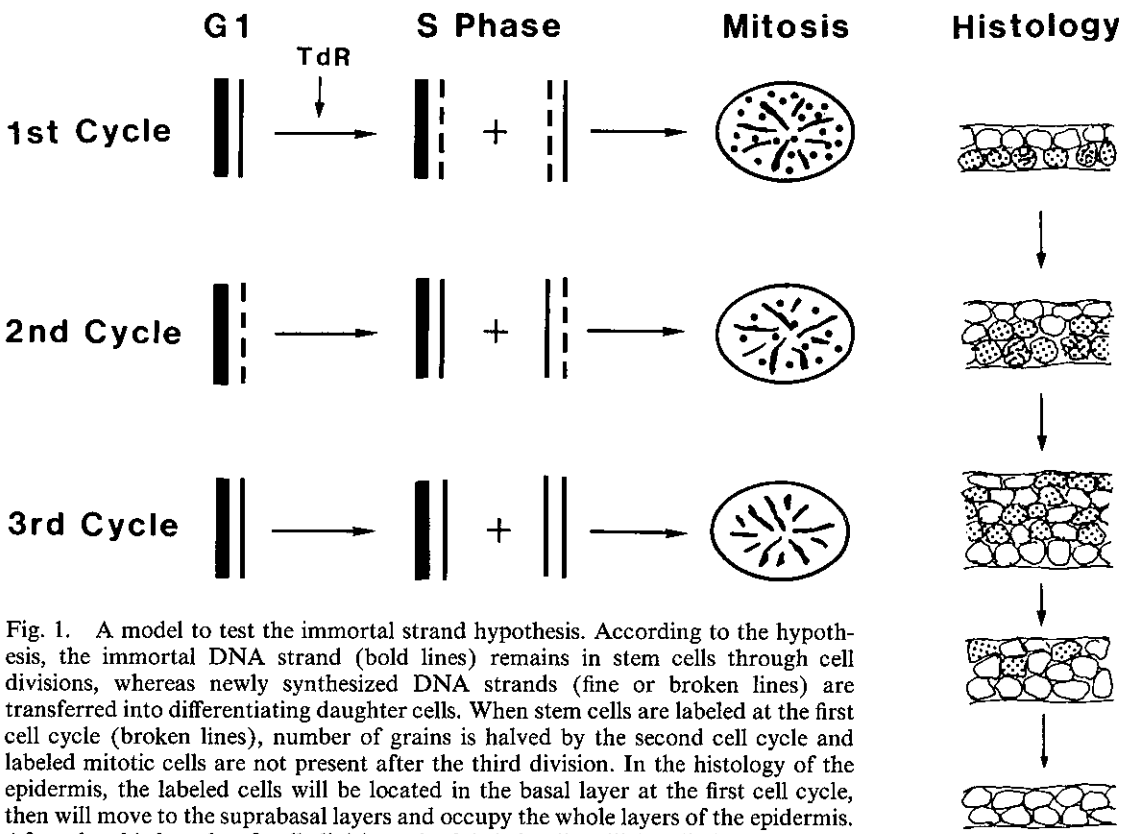

Fig. 1. A model to test the immortal strand hypothesis. According to the hypothesis, the immortal DNA strand (bold lines) remains in stem cells through cell divisions, whereas newly synthesized DNA strands (fine or broken lines) are transferred into differentiating daughter cells. When stem cells are labeled at the first cell cycle (broken lines), number of grains is halved by the second cell cycle and labeled mitotic cells are not present after the third division. In the histology of the epidermis, the labeled cells will be located in the basal layer at the first cell cycle, then will move to the suprabasal layers and occupy the whole layers of the epidermis. After the third cycle of cell division, the labeled cells will be eliminated due to terminal differentiation.

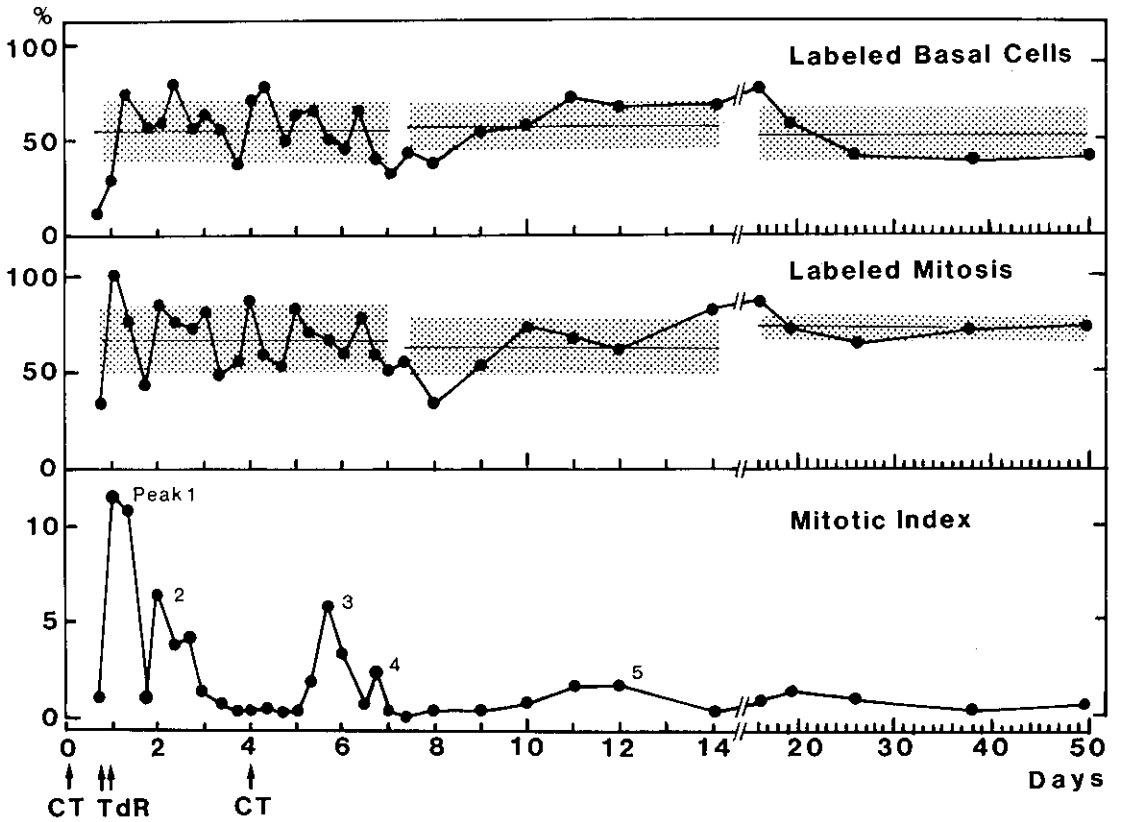

Fig. 2. Labeled basal cells (top), labeled mitotic cells (middle) and mitotic index (bottom) in the interfollicular epidermis of mice after injections of cholera toxin and [^{3}H]thymidine. Horizontal lines and meshed areas in the top and middle panels indicate average and SD, respectively, during the periods of days 1–7, 8–14 and 16–50. Arrows indicate the time of injection. CT, cholera toxin; TdR, [^{3}H]thymidine.

Pulse exposure to [^{3}H]thymidine will label only the newly synthesized strand. If selective segregation occurs, the labeled strand should be transferred into a non-stem daughter cell after two divisions. The number of grains in mitosis would be halved by the second cell division and no labeled mitosis would be seen after the third cell cycle. In the histology of the epidermis, labeled cells will be seen only in the basal layer at the first cell cycle. If the hypothesis is correct, the labeled cells will move to the suprabasal layers and eventually be eliminated. Thus, at least three cycles of cell division, preferably synchronous divisions, are needed to test this hypothesis.

Induction of cell divisions by cholera toxin The above conditions were obtained by subcutaneous injection of cholera toxin at a dose of 50 ng to the dorsal skin of mice using tongue forceps[5] on days 0 and 4. As shown in Fig. 2 (bottom panel), injection of cholera toxin evoked mitotic stimulation in the epidermis. The mitotic index of the basal cells reached a first peak at 24 h and a second peak at 48 h. The second injection of cholera toxin was found to induce a third peak on day 5 (138 h) and a fourth peak on day 6 (162 h), followed by a small and broad peak on day 11–12. Injection of the toxin on day 3, 5 or 7 induced small or broad peaks of mitosis (data not included).

Persistent presence of labeled basal cells The epidermal cells were labeled with [^{3}H]thymidine (10 μCi/mouse) at the first cycle of DNA synthesis and autoradiographs were prepared. Labeled cells were first found only in the basal layer where stem cells of the interfollicular epidermis are located (Fig. 3A). These labeled cells then moved to the suprabasal layers and were distributed throughout the hyperplastic epidermis after the second peak of mitosis, i.e. 48 h (Fig. 3B–G). Percentages of labeled basal cells did not change, remaining at 51–58%, during the observation period of 50 days (Fig. 2, top panel and Fig. 3). Such persistent presence of labeled cells together with their histological distribution suggests random segregation of the newly synthesized DNA strand.

Persistent presence of labeled mitotic cells Mitotic cells were arrested by intraperitoneal injection of demecolcin (50 μg/mouse) 3 h before killing. Labeled mitotic cells

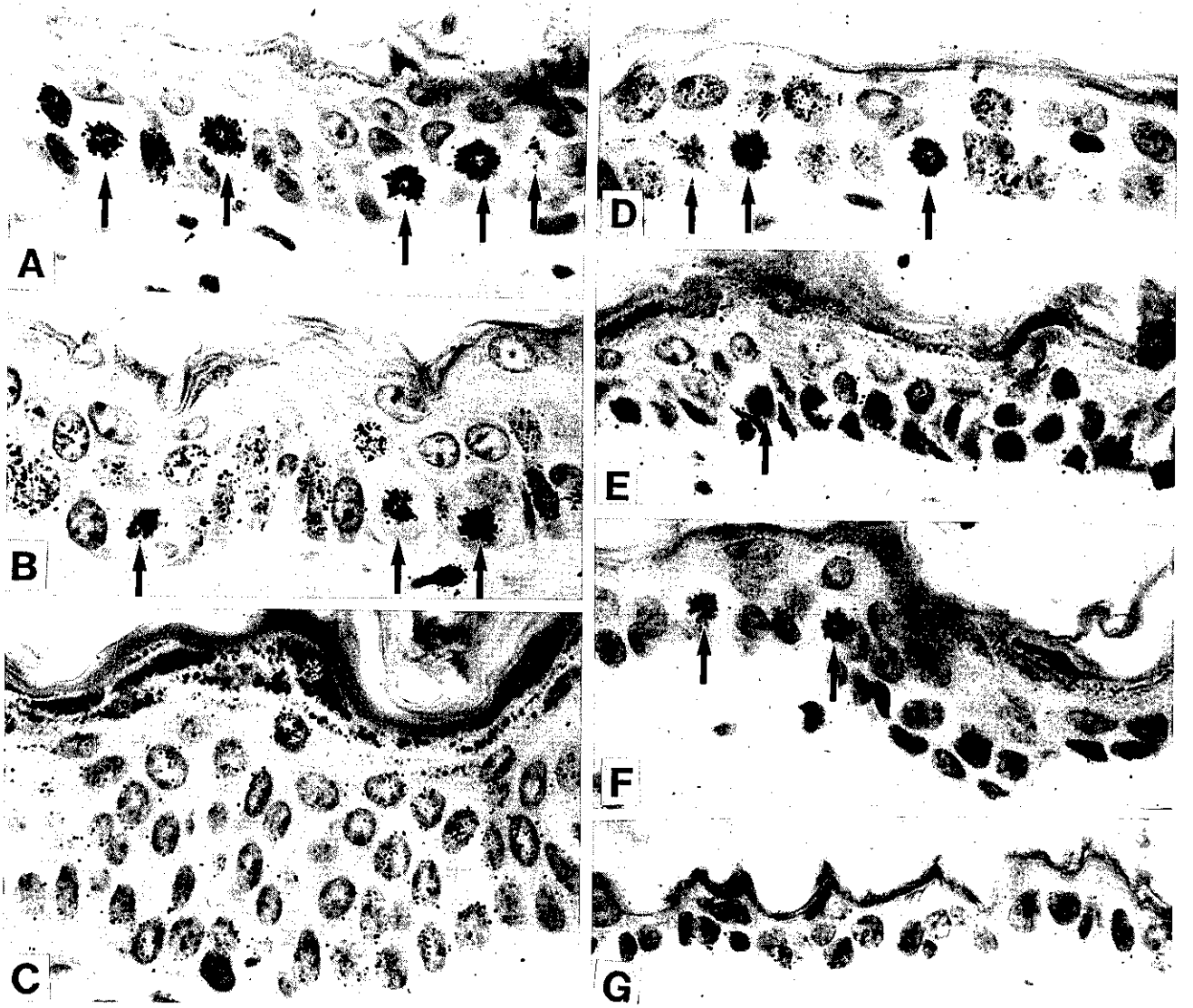

Fig. 3. Autoradiographs of the epidermis of mice. A, 24 h (peak 1 in Fig. 2 bottom panel); B, 48 h (peak 2); C, day 4; D, day 5 and 18 h (peak 3); E, day 6 and 18 h (peak 4); F, day 12 (peak 5); G, day 50. Note that at the first cycle of cell division (A, 24 h) only the basal cells were labeled but thereafter the labeled cells were found in the whole layer of the epidermis. Also note that the number of grains per mitosis decreased with time through cell divisions. Hyperplasia is most remarkable on day 4 (C); dark spots at the uppermost layers (stratum corneum and granulosum) are keratohyalin granules. Arrows indicate mitotic cells.

were also persistently present at 60–70% for 50 days, during which time there were 5 peaks of mitosis (Fig. 2, middle panel and Fig. 3). This observation does not fit well with the immortal strand hypothesis, because if selective segregation occurs, no labeled mitotic cells should be present after the third cycle of cell division according to the model shown in Fig. 1.

Logarithmic decrease of grains in mitotic cells Further evidence against this hypothesis is provided by the kinetics of decrease in the label. As shown in Fig. 4, the number of grains decreased exponentially through 5 cycles of division corresponding to peaks 1 to 5 in Fig. 2 (bottom panel). The rate of loss of the label at each round of cell division was estimated to be $48.8 \pm 11.4\%$

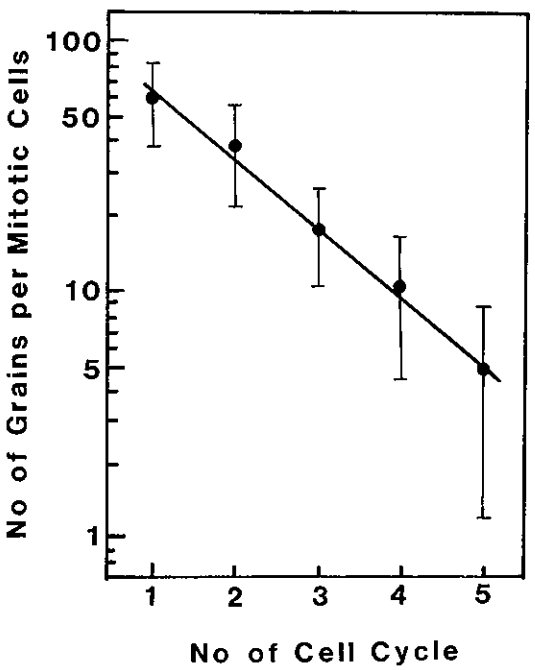

Fig. 4. Logarithmic decrease of the number of grains per mitotic cells through cell divisions. Numbers on the abscissa indicate the peaks of mitotic index shown in Fig. 2 bottom panel. Bars, SD; n, 20 mitotic cells.

(average and SD of the 5 cycles). This result indicates that DNA strands are transferred randomly to the two daughter cells. If the hypothesis were correct, the number of grains would be halved at the second division, then the grains would be eliminated.

DISCUSSION

The present study was undertaken to examine the immortal strand hypothesis proposed by Cairns in 1975,[2] one of the most difficult and controversial questions of stem cell proliferation. We could not obtain evidence in favor of this hypothesis. Persistent labeling of basal cells and basal mitotic cells and a logarithmic decrease of the number of grains over 5 cell cycles rather suggest random segregation of DNA strands in epidermal basal cells.

Because of the complexity of the question itself, there are some intrinsic factors to be considered in the present system: these include SCE, re-utilization of enucleated DNA and primitive stem cells in the epidermis. It is known that decay of tritium can induce SCE which may make uncertain the selective segregation of DNA strands, if it occurs. In the present study, we used a very low dose of [^{3}H]thymidine (necessitating a 3-month exposure in autoradiography), thus minimizing possible induction of SCE. Re-utilization of breakdown products of DNA from enucleated nuclei at the stratum corneum or from other tissues may also mask selective segregation. However, the exponential decrease in the number of grains shown in Fig. 4 suggests that enucleated DNA is not utilized again for DNA synthesis of stem cells. Furthermore, it is very unlikely that the incorporated thymidine molecules would be removed in the form of thymidine from nuclei and re-utilized. If tritium atoms are re-utilized, most of the radioactivity would be expected to be found in the cytosol, but grains were found exclusively on nuclei.

The presence of primitive stem cells in the epidermis is suggested from the results of surgical operation for skin grafting. Grafting of thin layers (about 0.4 mm) of skin containing the interfollicular epidermis and upper portions of the hair follicles results in skin without hairs at the site of grafting, while normal skin with hairs is recovered at the donor site of the graft. This result suggests that stem cells of the interfollicular epidermis, when eliminated, are supplied from the remaining portions of hair follicles where primitive stem cells may be located. Our present observation seems to apply to stem cells of the interfollicular epidermis.

We interpret the above observations on the assumption that cholera toxin stimulates DNA synthesis of stem cells under steady-state conditions in which the number of stem cells remains constant. Potten *et al.*[6] referred to such a situation as a "new strand labeling experiment." However, when stem cells are recruited following damage to tissues, for example, caused by radiation, the old immortal strand will be labeled. Under such circumstances, referred to as an "immortal strand labeling experiment,"[6] labeling will be halved by the second cell division and will remain at the same level thereafter. The results in Fig. 4 do not fit with either experimental model.

Only a few papers have been published concerning tests of the immortal strand hypothesis. Potten *et al.*[6] conducted "new strand labeling experiments" with tongue papilla and "immortal strand labeling experiments" with regenerating intestinal crypts after X-irradiation. Their results, though complicated and incomplete, were interpreted as evidence for the hypothesis. Potten[7] further reported that when a high dose of [^{3}H]thymidine was injected shortly after birth or after plucking of adult hairs at which melanocytes are undergoing replication, progressive depigmentation of hairs was observed at the site of the injection. This implies that the label is retained in the melanoblasts throughout the life time, resulting in killing of melanoblasts and thereby depigmentation of the hairs. Assuming that [^{3}H]thymidine is incorporated into the immortal strand, i.e. an immortal strand labeling experiment, this can be interpreted as evidence in favor of the hypothesis.

The presence of persistently labeled cells was reported in the epidermis and oral mucosa of mice and hamsters after labeling of newborn or adult animals with [^{3}H]-thymidine.[8-11] Although such label-retaining cells are conceived to be stem cells, these observations do not provide evidence to support or exclude the immortal strand hypothesis.

Using clonal markers of the colonic crypts, Griffiths *et al.*[12] and Winton *et al.*[13] demonstrated that stem cells are a target of somatic mutation and carcinogenesis by chemical carcinogens. Estimated rates of mutation in stem cells in these studies were very similar, i.e. 3.6×10^{-4} per crypt[12] after 8 weekly doses of dimethylhydrazine, a colon carcinogen and 5.1×10^{-4} per crypt[13] when mice were exposed to ethylnitrosourea *in utero*. DNA damage and resulting mutations seem to be memorized in stem cells for a long period. In two-stage cercinogenesis of mouse skin, tumors are produced when a promoter is applied to the initiated skin after a long intermission (68 weeks).[14] In atomic bomb survivors, solid tumors have developed in excess many years after exposure.[15]

All these and other experimental and epidemiological data suggest that DNA damage in stem cells can be memorized for many years. There are several possible mechanisms for memory of DNA damage in stem cells. These include point mutation, deletion and translocation of genes such as oncogenes. If these changes occur in stem cells, they will be memorized over cell divisions irrespective of the immortal strand hypothesis, thus indicating the importance of stem cells in carcinogenesis.

ACKNOWLEDGMENTS

We are grateful to Dr. Hiraku Takebe, Kyoto University and Drs. Lorenzo Tomatis and Ruggero Montesano, International Agency for Research on Cancer, Lyon, France, for their reading of the manuscript. The authors are also indebted to Dr. John Cairns, Harvard School of Public Health who read the manuscript and criticized at several points.

(Received March 22, 1989/Accepted May 12, 1989)

REFERENCES

1) Lajtha, L. G. Stem cell concepts. *Differentiation,* **14,** 23–34 (1979).
2) Cairns, J. Mutation selection and the natural history of cancer. *Nature,* **255,** 197–200 (1975).
3) Potten, C. S. (ed.) "Stem Cells, Their Identification and Characterisation" (1983). Churchill Livingstone, Edinburgh, London, Melbourne & New York.
4) Kuroki, T. Induction by cholera toxin of synchronous divisions *in vivo* in the epidermis resulting in hyperplasia. *Proc. Natl. Acad. Sci. USA,* **78,** 6958–6962 (1981).
5) Kuroki, T. Cholera toxin as a mitotic stimulator of epidermal cells *in vitro* and *in vivo*. *In* "Bacterial Diarrheal Diseases," ed. Y. Takeda and T. Miwatani, pp. 161–168 (1985). KTK Scientific Publishers, Tokyo.
6) Potten, C. S., Hume, W. J., Reid, P. and Cairns, J. The segregation of DNA in epithelial stem cells. *Cell,* **15,** 899–906 (1978).
7) Potten, C. S. Sensitivity of follicular melanoblasts in newborn mouse skin to tritiated thymidine: evidence for a long term retention of label. *Experientia,* **38,** 1464–1468 (1982).
8) Bickenbach, J. R. Identification and behavior of label-retaining cells in oral mucosa and skin. *J. Dent. Res.,* **60 (C),** 1611–1620 (1981).
9) Bickenbach, J. R. and Mackenzie, I. C. Identification and localization of label-retaining cells in hamster epithelia. *J. Invest. Dermatol.,* **82,** 618–622 (1984).
10) Bickenbach, J. R., McCutecheon, J. and Mackenzie, I. C. Rate of loss of tritiated thymidine label in basal cells in mouse epithelial tissues. *Cell Tissue Kinet.,* **19,** 325–333 (1986).
11) Morris, R. J., Fischer, S. M. and Slaga, T. J. Evidence that the centrally and peripherally located cells in the murine epidermal proliferative unit are two distinct cell populations. *J. Invest. Dermatol.,* **84,** 277–281 (1985).
12) Griffiths, D. F. R., Davies, S. J., Williams, D., Williams, G. T. and Williams, E. D. Demonstration of somatic mutation and colonic crypt clonality by X-linked enzyme histochemistry. *Nature,* **333,** 461–463 (1988).
13) Winton, D. J., Blount, M. A. and Ponder, B. A. J. A clonal marker induced by mutation in mouse intestinal epithelium. *Nature,* **333,** 463–466 (1988).
14) Stenbäck, F., Peto, R. and Schubik, P. Initiation and promotion at different ages and doses in 2200 mice. I. Methods, and the apparent persistence of initiated cells. *Br. J. Cancer,* **44,** 1–14 (1981).
15) Kato, H. Cancer mortality. *Gann Monogr. Cancer Res.,* **32,** 53–74 (1986).