Jpn. J. Cancer Res. 85, 692–698, July 1994

Increased Cell Proliferation of Azoxymethane-induced Aberrant Crypt Foci of Rat Colon

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Aberrant crypt foci (ACF) were induced in the colon of F344 rats by s.c. injection of azoxymethane (AOM) twice in a three day-interval and examined after 4 and 12 weeks. The number and crypt multiplicity of ACF in each section of rat colon increased during this period. Histologically, aberrant crypts consisted of proliferating atypical epithelial cells. Cell proliferation of ACF consisting of 4 aberrant crypts [ACF(4)] and 2 aberrant crypts [ACF(2)], and normal crypts in the colon of rats treated with AOM [normal crypts/AOM(+)] or saline [normal crypts/AOM(−)] was investigated by measurement of the mitotic index, proliferating cell nuclear antigen-labeling index (PCNA-LI), and 5-bromo-2′-deoxyuridine-labeling index (BrdU-LI). All three parameters of the cell proliferative activity of ACF(4) were higher than those of normal crypts/AOM(+) and normal crypts/AOM(−). The PCNA-LI and BrdU-LI in ACF(2) were the same as those in ACF(4). These findings suggest that ACF have increased cell proliferative activity. The correlation of these three parameters confirmed that the PCNA-LI is also a useful parameter for evaluating cell proliferative activity in ACF. The presence of many cells stained by PCNA in the upper portion of ACF suggested that ACF have more G_1 phase cells, which readily respond to mitogenic stimulation, than G_0 phase cells, which are predominant in normal crypts.

Key words: Cell proliferation — Aberrant crypt focus — PCNA — BrdU — Colon

Colorectal cancer is one of the main causes of death from cancer in Western countries,[1] and this may also soon be the case in Asian countries due to rapid Westernization of the life style.[1] Thus, studies on colorectal carcinogenesis are very important. For development of methods for preventing colon carcinogenesis it is essential to establish suitable biomarkers for detection of conditions of high risk of carcinogenesis. Since Bird *et al.* reported the existence of aberrant crypts in the murine colon treated with a colon carcinogen,[2] much attention has been paid to them as a putative early lesion and a useful biomarker of colon carcinogenesis.

Morphologically, aberrant crypts are distinguishable from normal crypts by their larger size and the more elliptical shape of the luminal opening, with a thicker lining of epithelial cells.[2] Genetically, expressions of c-*fos*[3] and *ras*,[4] as well as Ki-*ras* point mutations,[5] are increased in aberrant crypt foci (ACF). Moreover, enzyme histochemical studies have indicated that hexosaminidase activity is altered in human colonic carcinoma[6,7] and decreased in rat ACF.[8] In human cases, ACF are more frequent in the colon of patients with colorectal cancer than in those without colon cancer, and also in subjects with a predisposition for cancer.[9] The ACF found in the colon of patients with familial adenomatous polyposis were reported to be histologically more dysplastic than those in subjects with benign diseases such as diverticular disease.[10]

In addition to morphologic analyses, it is important to investigate the cell proliferative kinetics of ACF for further understanding of the nature of those minute lesions and their possible involvement in colorectal cancer development. This has been examined by studies on their labeling index in rats by McLellan *et al.*[11] and in humans by Roncucci *et al.*[12] However, the safety of using 5-bromo-2′-deoxyuridine (BrdU), which is directly incorporated into DNA, has not yet been confirmed, so the BrdU labeling index (BrdU-LI) is unsuitable for studies on cell proliferation in humans. Therefore, in this work we tried to establish a better method for studying cell proliferation in ACF.

Proliferating cell nuclear antigen (PCNA), also called cyclin,[13] was discovered through an autoantibody in the serum of a patient with systemic lupus erythematosus that specifically reacted with the nuclei of proliferating cells.[14] Later it was identified as an auxiliary protein of 36 kDa[15] of DNA polymerase-δ.[16,17] It can be used as a proliferation index of a broader spectrum of cells than other parameters,[18] and has been reported to be a promising predictor of the prognosis of patients with malignant diseases such as gastric cancer[19] and hemangiopericytoma.[20] In this study we analyzed the localization of

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cells with increased proliferative activity in ACF, the change in the ability to proliferate according to crypt multiplicity and the induction period of ACF by following the mitotic index (MI), BrdU incorporation, and PCNA staining, in AOM-treated rats.

MATERIALS AND METHODS

Animals and treatment Five-week-old male F344 rats (Charles River Japan, Atsugi) were housed in wire cages in a room, with a 12 h light-dark cycle at 23°C, and were allowed free access to water and food (CE-2, CLEA, Tokyo). One group of 27 rats was treated s.c. twice in a three day-interval with 15 mg/kg body weight of azoxymethane (AOM) (Sigma, St. Louis, MO) and a control group of 13 rats was treated similarly with saline. Seventeen of the AOM-treated rats and 8 of the saline-treated rats were killed after 4 weeks and the remaining rats in each group were killed after 12 weeks. Seven AOM-treated rats and 3 saline-treated rats killed in week 4 were treated i.p. with BrdU at a dose of 100 mg/kg body weight 1 h before death.[21]

The colons were promptly removed and fixed overnight in 10% buffered formalin at 4°C. After staining with 0.2% methylene blue in phosphate-buffered saline (PBS), ACF were observed by light microscopy.[2] We defined the rectum as the segment 2 cm proximal to the anus, and divided the remaining colon into three segments, named the distal colon, the middle colon, and the proximal colon. We counted the number of ACF in each segment. We also counted the number of aberrant crypts comprising each focus, which was defined as "crypt multiplicity."

Tiny portions of distal colonic mucosal tissue containing ACF consisting of 4 aberrant crypts [ACF(4)] and 2 aberrant crypts [ACF(2)] were removed with a Pasteur pipette under a microscope. Tissues of the control group were taken from the distal colon. These tissues were embedded in paraffin and serial vertical sections were made for hematoxylin and eosin (H-E) staining and immunostaining.

Mitotic index Mitotic figures were counted in vertical sections through the center of crypts. The MI was calculated as follows:

$$MI = \frac{\text{number of mitoses in the crypt}}{\text{total number of nuclei in the crypt}} \times 1000$$

Immunostaining for PCNA and BrdU PCNA-staining was performed by the avidin-biotin complex (ABC) method.[22] Tissue sections were deparaffinized with xylene and a graded ethanol series, and incubated with methanol containing 0.3% hydrogen peroxide (H_2O_2) for 30 min to block endogenous peroxidase activity. They were then incubated with 10% normal horse serum at room temperature for 30 min to block background staining, and incubated overnight at 4°C with mouse monoclonal antibody to PCNA (PC10, Novocastra, Newcastle, UK) diluted 1:200. Then they were incubated for 30 min at room temperature with biotinylated horse anti-mouse IgG (Vector, Burlingame, CA), and with ABC (Vector) at 1:25 dilution. Each step was followed by washing with PBS. Peroxidase activity was visualized by treatment with 0.02% diaminobenzidine tetrahydrochloride (DAB)/0.05 *M* Tris-HCl (pH 7.6) containing 0.05% hydrogen peroxide. Then nuclei were counterstained with hematoxylin or methylgreen. BrdU was immunostained by the method described previously[21] with some modifications, simultaneously with PCNA staining in serial sections of colonic tissues obtained from seven rats treated i.p. with BrdU. This allowed examination of the correlation between the PCNA-labeling index (PCNA-

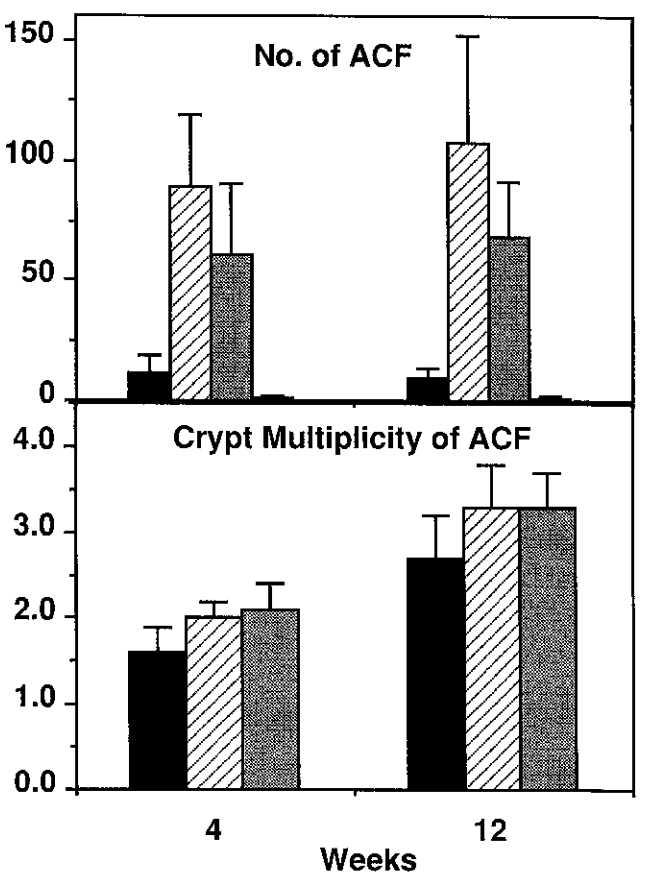

Fig. 1. Incidence and crypt multiplicity of ACF in each segment of rat colon (■: rectum, ▨: distal colon, ▩: middle colon, □: proximal colon) at 4 and 12 weeks after the first AOM treatment. The incidence and crypt multiplicity of ACF increased during the experiment.

LI) and BrdU-LI. Endogenous peroxidase was blocked by treatment with methanol containing H_2O_2 as described above, and then tissue sections were treated with 4 *N* HCl at 37°C for 20 min and neutralized with boric acid-borax buffer (pH 7.6). Subsequently, they were incubated with 0.02% actinase E (Kaken, Tokyo). After incubation with 10% normal horse serum at room temperature for 30 min, they were incubated overnight at 4°C with mouse

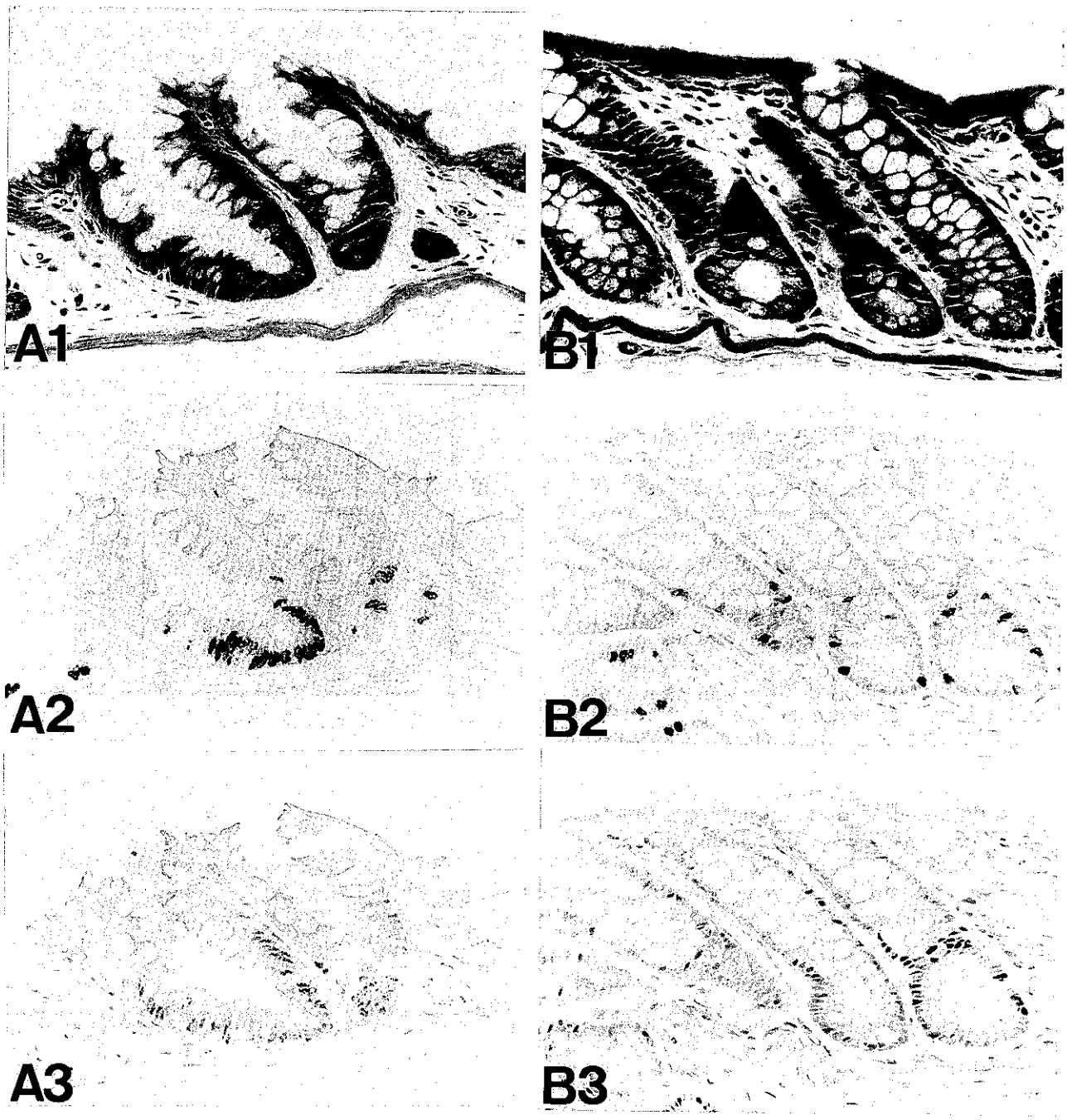

Fig. 2. Serial vertical sections of ACF (2A-1, 2, 3) and normal crypts (2B-1, 2, 3). A-1 and B-1 are stained with H-E. Aberrant crypts consisted of proliferating atypical epithelial cells. A-2 and B-2 were stained with BrdU. A-3 and B-3 were stained with PCNA. The numbers of PCNA or BrdU-stained cells in ACF were more than those in normal crypts. PCNA-stained cells overlapped most of the BrdU-stained cells. (×937)

monoclonal antibody to BrdU (Dakopatts, Denmark) diluted 1:500. The subsequent steps were the same as those for PCNA staining.

Aberrant crypts and normal crypts centrally sectioned from their surface to their bottom were observed to determine PCNA-LI and BrdU-LI. Considering the well known properties of PCNA,[23-26] we counted all nuclei that were positive for PCNA to various degrees. The PCNA-LI and BrdU-LI were expressed as numbers of stained nuclei calculated as percentages (%) of total nuclei in a crypt.

Statistical analysis Statistical analysis of data was performed by using Student's t test. A value of $P \leq 0.05$ was considered as statistically significant.

RESULTS

Time-dependent change in number and multiplicity of ACF and their distribution The numbers of ACF induced by AOM were 163.3 ± 50.9/rat at 4 weeks and 186.1 ± 52.7/rat at 12 weeks after the first AOM treatment. The values of crypt multiplicity of ACF induced by AOM were 2.00 ± 0.18 at 4 weeks and 3.28 ± 0.40 at 12 weeks after the first AOM treatment. The number and crypt multiplicity of ACF increased in all regions of the colon of AOM-treated animals during the experiment, but no ACF was found in the control groups at 4 or 12 weeks (Fig. 1).

Histological findings Histologically, individual aberrant crypts consisted of proliferating atypical epithelial cells. Mitotic figures were present mainly in the middle portion of aberrant crypts, in contrast to those in normal crypts, which were mainly found in the bottom of crypts (Fig. 2A-1, 2B-1).

Cell proliferation in ACF Immunohistochemical examination showed that BrdU-positive cells were located in the bottom of normal crypts. In contrast, BrdU-positive cells in ACF were located in upper parts, besides the bottom of crypts (Fig. 2A-2, 2B-2). Similar findings were observed on staining with PCNA (Fig. 2A-3, 2B-3). PCNA-positive cells overlapped most of BrdU-positive cells and were also present in the upper part of crypts compared to the BrdU-stained cells.

The MI of ACF(4) [$14.0 \pm 11.8(\times 10^{-3})$] was significantly higher than those of normal crypts in the colon of rats treated with AOM [normal crypts/AOM(+)] [$5.4 \pm 8.2(\times 10^{-3})$] ($P<0.001$) and of normal crypts in the colon of rat treated with saline [normal crypts/AOM(−)] [$7.0 \pm 10.5(\times 10^{-3})$] ($P<0.01$). There was no significant difference between the MI of normal crypts/AOM(+) and normal crypts/AOM(−). The PCNA-LI of ACF(4) [33.7 ± 13.6(%)] was significantly higher than those of normal crypts/AOM(+) [22.1 ± 10.3(%)] ($P<0.001$) and normal crypts/AOM(−) [22.0 ± 9.6(%)] ($P<0.001$). There was no significant difference between the PCNA-LI's of normal crypts/AOM(+) and normal crypts/AOM(−). The time-dependent change in cell proliferation activity of ACF was examined by comparing the MI's and PCNA-LI's at different times after the first carcinogen treatment. ACF with the same crypt multiplicity (4) had the same cell proliferative activity at 4 weeks and 12 weeks after the first carcinogen treatment (Table I).

The cell proliferative activity of ACF was further analyzed in terms of the crypt multiplicity of ACF 4 weeks after the first carcinogen treatment. The PCNA-LI and BrdU-LI of ACF(4) were almost the same as those of ACF(2). The PCNA-LI of ACF(2) was significantly higher than those of normal crypts/AOM(+) and normal crypts/AOM(−) ($P<0.05$, 0.01). The BrdU-LI of ACF(2) was also significantly higher than those in normal crypts/AOM(+) and normal crypts/AOM(−) ($P<0.01$, 0.01) (Table II). Similarly significant differences were recognized between the BrdU-LI of ACF(4) and those of normal crypts/AOM(+) and normal crypts/AOM(−) ($P<0.001$, 0.001). The significant differences between the PCNA-LI of ACF(4) and those of normal crypts/AOM(+) and normal crypts/AOM(−)

Table I. MI and PCNA-LI in ACF and Normal Crypts at 4 and 12 Weeks after the First Carcinogen Treatment

Tissue sample	Weeks	Mitotic index ($\times 10^{-3}$)	n	PCNA-LI (%)	n
ACF	4	15.6 ± 13.0	17	35.5 ± 14.7	19
	12	12.5 ± 10.7	17	30.7 ± 11.5	14
Normal crypt/AOM(+)	4	6.8 ± 9.0	31	20.4 ± 9.1	14
	12	4.0 ± 7.1	31	23.9 ± 12.2	11
Normal crypt/AOM(−)	4	8.3 ± 11.7	30	26.1 ± 10.2	20
	12	5.7 ± 9.2	30	18.0 ± 7.0	20

ACF consisted of 4 aberrant crypts. n, number of samples.

Table II. Correlation between PCNA-LI and BrdU-LI in ACF(2, 4) and Normal Crypts at 4 Weeks after the First Carcinogen Treatment

Tissue sample	PCNA-LI (%)	n	BrdU-LI (%)	n
ACF(2)	29.0±11.7[a)]	17	18.8±10.6[a)]	17
ACF(4)	29.3±11.7[a)]	20	21.0±10.6[a)]	25
NC·AOM(+)	20.7±9.3[b)]	18	10.2±4.0[b)]	16
NC·AOM(−)	18.4±4.3[b)]	18	6.3±2.4[c)]	18

a, b, c) Means with different superscripts in the same column are significantly different at $P<0.05$.
n, number of samples.

were confirmed in this experiment, but the BrdU-LI in normal crypts/AOM(+) was also significantly higher than that in normal crypts/AOM(−) ($P<0.01$) (Table II).

DISCUSSION

In the present study, the number and crypt multiplicity of ACF in whole regions of the colon increased during the 12-week period after the first treatment with carcinogen. A similar observation has been reported by McLellan *et al.*[27] Increase in crypt multiplicity may be one of the characteristics of clonal expansion in ACF. Therefore, we investigated the cell proliferative activity in ACF in the distal colon, where ACF were most frequently induced.

We observed clear enhancement of cell proliferation in rat ACF using three parameters of cell proliferation, the MI, BrdU-LI, and PCNA-LI. These results are consistent with previous findings obtained with [^{3}H]thymidine.[12] BrdU is a good parameter for evaluating cell proliferation because only S phase cells are labeled. PCNA-staining labels cells in both the G_1 and S phases.[16, 28] In general, good correlations between the PCNA-LI and BrdU-LI have been found in various organs,[29, 30] but in some forms of neoplasms, including breast and gastric cancers and cultured cell lines, the correlation between PCNA expression and cell proliferation is not good.[18] In this investigation, the PCNA-LI was found to be a useful and convenient marker for evaluating cell proliferative activity in ACF, and to correlate well with the BrdU-LI and MI. Although the BrdU-LI is one of the most reliable parameters of cell proliferation, BrdU is incorporated into DNA of proliferating cells. BrdU has been classified as a potential carcinogen[30] and the safety of BrdU-labeling of human cells has not yet been confirmed. Thus, the PCNA-LI is a better parameter to use in studying human ACF.

A significant difference was found between the BrdU-LI in normal crypts/AOM(+) and that in normal crypts/AOM(−), but not between their PCNA-LI. In spite of having the same morphological appearance as normal crypts/AOM(−), normal crypts/AOM(+) may have increased proliferative activity. But this difference could also be due to the difference between the phases of PCNA-positive cells and BrdU-positive cells, so further investigations are required, such as on the effect of changing the time of examination after BrdU treatment.

From the present study, we also concluded that the ACF are foci of abnormal colon epithelium with increased cell proliferation for the following reasons. First, the crypt multiplicity of ACF gradually increased. Second, the PCNA-LI and BrdU-LI of ACF(2) and ACF(4) were almost the same, indicating that on formation of ACF the cell proliferative activity increased. Moreover, the fact that ACF(4) induced for 4 weeks have the same cell proliferative activity indicated that ACF have increased cell proliferative activity irrespective of crypt multiplicity and period for induction of ACF. In this study, we examined PCNA-LI in addition to BrdU-LI of ACF. The majority of PCNA-positive cells were also BrdU-positive. PCNA is known to stain cells in both the G_1 and S phases. The PCNA-positive cells were mainly localized in the lower part of normal crypts. But some cells in the upper portion of ACF were stained by PCNA but not by BrdU, and most of these cells were concluded to be in the G_1 phase. Cells in the G_1 phase undergo the next cell division more readily than those in the G_0 phase in the presence of a growth stimulant. Moreover, cell proliferation is supposed to be essential for fixation of the genetic changes required for tumorigenesis. Therefore, ACF have not only increased proliferative activity, but also a higher possibility of genetic changes than normal crypts.

Increased cell proliferation has been proposed to be a biomarker of increased susceptibility to gastrointestinal cancer.[31, 32] This investigation indicated that ACF may be used as a biomarker of colorectal cancer.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for Cancer Research, a grant from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, and a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan. We thank Mrs. M. Takahashi for constructive criticism.

(Received January 17, 1994/Accepted March 22, 1994)

REFERENCES

1) "World Health Statistics Annual 1991," pp. 90–287 (1991). World Health Organization, Geneva.
2) Bird, R. P. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.*, **37**, 147–151 (1987).
3) Stopera, S. A., Davie, J. R. and Bird, R. P. Colonic aberrant crypt foci are associated with increased expression of c-*fos*: the possible role of modified c-*fos* expression in preneoplastic lesions in colon cancer. *Carcinogenesis*, **13**, 573–578 (1992).
4) Stopera, S. A. and Bird, R. P. Expression of *ras* oncogene mRNA and protein in aberrant crypt foci. *Carcinogenesis*, **13**, 1863–1868 (1992).
5) Stopera, S. A., Murphy, L. C. and Bird, R. P. Evidence for a *ras* gene mutation in azoxymethane-induced colonic aberrant crypts in Sprague-Dawley rats: earliest recognizable precursor lesion of experimental colon cancer. *Carcinogenesis*, **13**, 2081–2085 (1992).
6) Brattain, M. G., Kimball, P. M. and Pretlow, T. G. β-Hexosaminidase isozymes in human colonic carcinoma. *Cancer Res.*, **37**, 731–735 (1977).
7) Brattain, M. G. and Pretlow, T. G. Cell fraction of large bowel cancer. *Cancer*, **40**, 2479–2486 (1977).
8) Pretlow, T. P., O'Riordan, M. A., Kolman, M. F. and Jurcisek, J. A. Colonic aberrant crypts in azoxymethane-treated F344 rats have decreased hexosaminidase activity. *Am. J. Pathol.*, **136**, 13–16 (1990).
9) Pretlow, T. P., Barrow, B. J., Ashton, W. S., O'Riordan, M. A., Pretlow, T. G., Jureisek, J. A. and Stellato, T. A. Aberrant crypts: putative preneoplastic foci in human colonic mucosa. *Cancer Res.*, **51**, 564–567 (1991).
10) Roncucci, L., Stamp, D., Medline, A., Cullen, J. B. and Bruce, W. R. Identification and quantification of aberrant crypt foci and microadenomas in the human colon. *Hum. Pathol.*, **22**, 287–294 (1991).
11) McLellan, E. A., Medline, A. and Bird, R. P. Dose response and proliferative characteristics of aberrant crypt foci: putative preneoplastic lesions in rat colon. *Carcinogenesis*, **12**, 2093–2098 (1991).
12) Roncucci, L., Pedroni, M., Fante, R., Di Gregorio, C. and Ponz de Leon, M. Cell kinetic evaluation of human colonic aberrant crypts. *Cancer Res.*, **53**, 3726–3729 (1993).
13) Mathews, M. B., Bernstein, R, M., Franza, B. R., Jr. and Garrels, J. I. Identity of the proliferating cell nuclear antigen and cyclin. *Nature*, **309**, 374–376 (1984).
14) Miyachi, K., Fritzler, M., Jr. and Tan, E. M. Autoantibody to nuclear antigen in proliferating cells. *J. Immunol.*, **121**, 2228–2234 (1978).
15) Bravo, R., Fey, S. J., Bellatin, J., Larsen, P. M., Arevalo, J. and Celis, J. E. Identification of a nuclear and a cytoplasmic polypeptide whose relative proportions are sensitive to changes in the rate of cell proliferation. *Exp. Cell Res.*, **136**, 311–319 (1981).
16) Bravo, R., Frank, R., Blundell, P. A. and MacDonald-Bravo, H. Cyclin/PCNA is the protein of DNA polymerase-δ. *Nature*, **326**, 515–517 (1987).
17) Prelich, G., Tan, C.-K., Kostura, M., Mathews, M. B., So, A. G., Downey, K. M. and Stillman, B. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. *Nature*, **326**, 517–520 (1987).
18) Hall, P. A., Levison, D. A., Woods, A. L., Yu, C. C.-W., Kellock, D. B., Watkins, J. A., Barnes, D. M., Gillet, C. E., Camplejohn, R., Dover, R., Waseem, N. H. and Lane, D. P. Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. *J. Pathol.*, **162**, 285–294 (1990).
19) Jain, S., Flipe, N. I., Hall, P. A., Waseen, N. H., Lane, D. P. and Levison, D. A. Application of proliferating cell nuclear antigen (PCNA) immunostaining in gastric cancer. *J. Pathol.*, **161**, 351 (1990).
20) Yu, C. C.-W., Hall, P. A., Fletcher, C. D. M., Camplejohn, R., Waseem, N. H., Lane, D. P. and Levison, D. A. Immunohistochemical staining with a monoclonal antibody to proliferating cell nuclear antigen may be a good indicator of prognosis in hemangiopericytomas. *J. Pathol.*, **161**, 342 (1990).
21) Tiwawech, D., Hasegawa, R., Kurata, Y., Tatematsu, M., Shibata, M., Thamavit, W. and Ito, N. Dose-dependent effects of 2-acetylaminofluorene on hepatic foci development and cell proliferation in rats. *Carcinogenesis*, **12**, 985–990 (1991).
22) Hsu, S.-M., Raine, L. and Fanger, H. A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. *Am. J. Clin. Pathol.*, **75**, 734–738 (1981).
23) Foley, J., Ton, T., Maronpot, R., Butterworth, B. and Goldsworthy, T. L. Proliferating cell nuclear antigen (PCNA): comparison to tritiated thymidine (^{3}H-TdR) as a marker of proliferating hepatocytes in rats. *Environ. Health Perspect.*, in press.
24) Hall, P. A. and Levison, D. A. Review: assessment of cell proliferation in histological material. *J. Clin. Pathol.*, **43**, 184–192 (1990).
25) Bravo, R. and MacDonald-Bravo, H. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J. Cell Biol.*, **105**, 1549–1554 (1987).
26) Kurki, P., Vandelaan, M., Dolbeare, F., Gray, J. and Tan, E. M. Expression of proliferating cell nuclear antigen (PCNA)/cyclin during the cell cycle. *Exp. Cell Res.*, **166**, 209–219 (1986).
27) McLellan, E. A., Medline, A. and Bird, R. P. Sequential analyses of the growth morphological characteristics of aberrant crypt foci: putative preneoplastic lesions. *Cancer*

Res., **51**, 5270–5274 (1991).
28) Takasaki, Y., Deng, J. S. and Tan, E. M. A nuclear antigen associated with cell proliferation and blast transformation — its distribution in synchronized cells. *J. Exp. Med.*, **154**, 1899–1909 (1981).
29) Foley, J. F., Dietrich, D. R., Swenberg, J. A. and Maronpot, R. R. Detection and evaluation of proliferating cell nuclear antigen (PCNA) in rat tissue by an improved immunohistochemical procedure. *J. Histotechnol.*, **14**, 237–241 (1991).
30) Coltrera, M. D. and Gown, A. M. PCNA/cyclin expression and BrdU uptake define different subpopulations in different cell lines. *J. Histochem. Cytochem.*, **39**, 23–30 (1991).
31) Lipkin, M. Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res.*, **48**, 235–245 (1988).
32) Lippman, S. M., Lee, J. S., Lotan, R., Hittelman, W., Wargovich, M. J. and Hong, W. K. Biomarkers as intermediate end points in chemoprevention trials. *J. Natl. Cancer Inst.*, **82**, 555–560 (1990).