

## Development and Distribution of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP)-induced Aberrant Crypt Foci in the Rat Large Intestine

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Aberrant crypt foci (ACF) are generally considered to be preneoplastic lesions for colon cancer. To assess their induction by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a colon carcinogen, we performed a sequential study of ACF morphology and localization. F344 male rats were given PhIP, and methylene blue-stained colon epithelium and isolated crypts were analyzed at weeks 12, 25, 50, and 75. Each crypt was classified into 2 groups, “single” with round bottoms and “bifurcating” displaying V-shaped clefts (indicating proliferation). In combination with the number of crypts in an ACF, this classification was a good indicator for the generation of ACF in line with the fission mechanism of growth. Increasing numbers of crypts in ACF through weeks 12 to 75 and decreased percentages of ACF with bifurcating crypts at the late time points indicated that proliferation of crypts occurs predominantly during the early stages. The distribution pattern showed a significant shift ( $P < 0.000005$ ) from the distal to the proximal part of the large intestine between weeks 25 and 50. Adenocarcinomas were first found to develop at week 50 in the ascending colon and cecum where bifurcating crypts were generally lacking at weeks 12 and 25. These data suggest the existence of (1) proliferating ACF which contains bifurcating crypt(s) and (2) quiescent or senescent ACF which consists of only single crypts.

Key words: PhIP — Aberrant crypt foci — Crypt isolation technique — Bifurcating crypts

Various mutagens and carcinogens are contained in the diet. Some of these, such as heterocyclic amines (HCAs), are produced in broiled meat and fish during cooking.<sup>1)</sup> Among several HCAs, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most abundant<sup>2)</sup> and considered one of the most important carcinogens taken in the daily diet. PhIP has been found to be more mutagenic than other heterocyclic amines in cultured mammalian cells in spite of its relatively low mutagenicity in the *Salmonella typhimurium* assay,<sup>3)</sup> and is known to induce aberrant crypt foci (ACF)<sup>4)</sup> and adenocarcinoma<sup>5)</sup> in the large intestine of F344 rats. Various oncogenes and tumor suppressor genes including those involved in the adenomatous polyposis coil (APC)/ $\beta$ -catenin pathway<sup>6,7)</sup> have been proposed to be candidate targets in PhIP-induced colon tumorigenesis.

ACF were firstly found in methylene blue-stained whole-mount preparations of colon epithelia in experimental animals and proposed as putative preneoplastic lesions of colorectal carcinomas.<sup>8)</sup> They are induced by administration of colon carcinogens in a dose-dependent manner.<sup>9)</sup> Histopathological assessment has shown that they may be indistinguishable from normal or adenomatous with marked cellular atypia in rats,<sup>10)</sup> but they generally have an

elevated proliferation index.<sup>11)</sup> The majority of ACF are located towards the rectal end,<sup>9)</sup> coinciding well with the distribution of azoxymethane (AOM)-induced colon tumors.<sup>12)</sup> Thus, there is compelling evidence that ACF are preneoplastic and give rise to colon cancers.

In humans, it has been reported that ACF are found mainly in the sigmoid colon and rectum.<sup>13)</sup> However, colorectal carcinomas also emerge in the proximal regions.<sup>14)</sup> Furthermore, Hardman *et al.*<sup>15)</sup> indicated that the number of ACF is not a reliable quantitative biomarker for the incidence of colon cancer.

Many of the actual processes involved in colon tumorigenesis are still unclear. We have successfully applied a crypt isolation technique for the isolation of ACF and classified each crypt into “single” or “bifurcating” according to the shape of the bottom of the crypt,<sup>16,17)</sup> reflecting the stage of development. In the present study, we analyzed PhIP-induced development of ACF through to colon tumors, concentrating on the distribution of ACF and percentages of bifurcating crypts.

### MATERIALS AND METHODS

**Animals** Three-week-old male Fisher rats were purchased from Charles River Japan Inc. (Kanagawa) and maintained on basal diet (Oriental NMF, Oriental Yeast Co., Tokyo)

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and water *ad libitum* in plastic cages in an air-conditioned room at 24°C with a 12 h light-12 h dark cycle.

**Experimental protocol** PhIP HCl (Nard Institute, Osaka) was dissolved at 20 mg/ml in water and 100 mg/kg body

weight was administered intragastrically 3 times a week for 7 weeks. Water alone was given to the control group. Ten rats each from the PhIP group and 5 each from the control group were killed at 12, 25, 50, and 75 weeks.

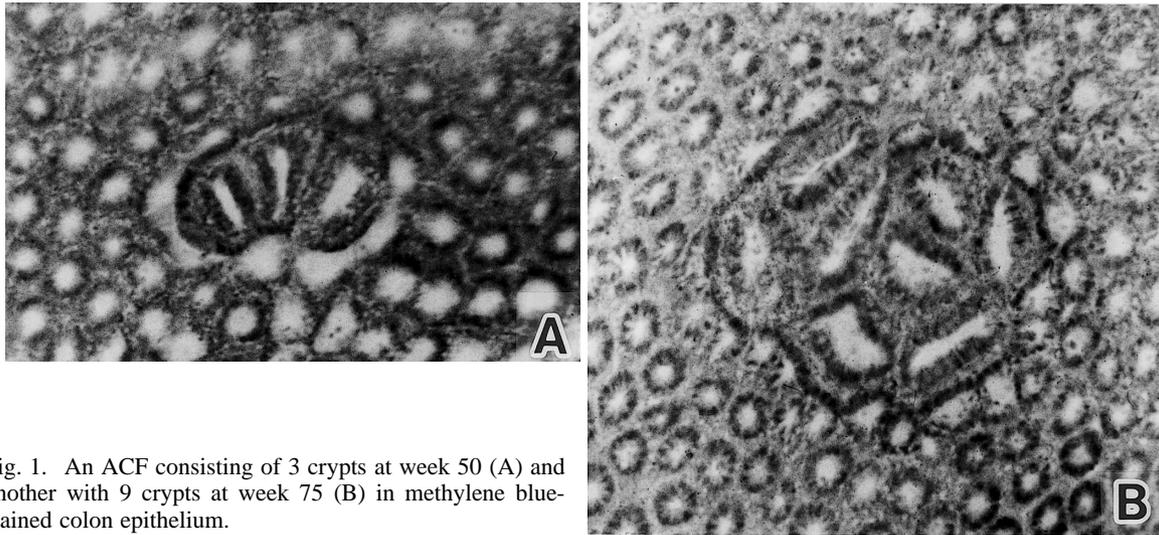


Fig. 1. An ACF consisting of 3 crypts at week 50 (A) and another with 9 crypts at week 75 (B) in methylene blue-stained colon epithelium.

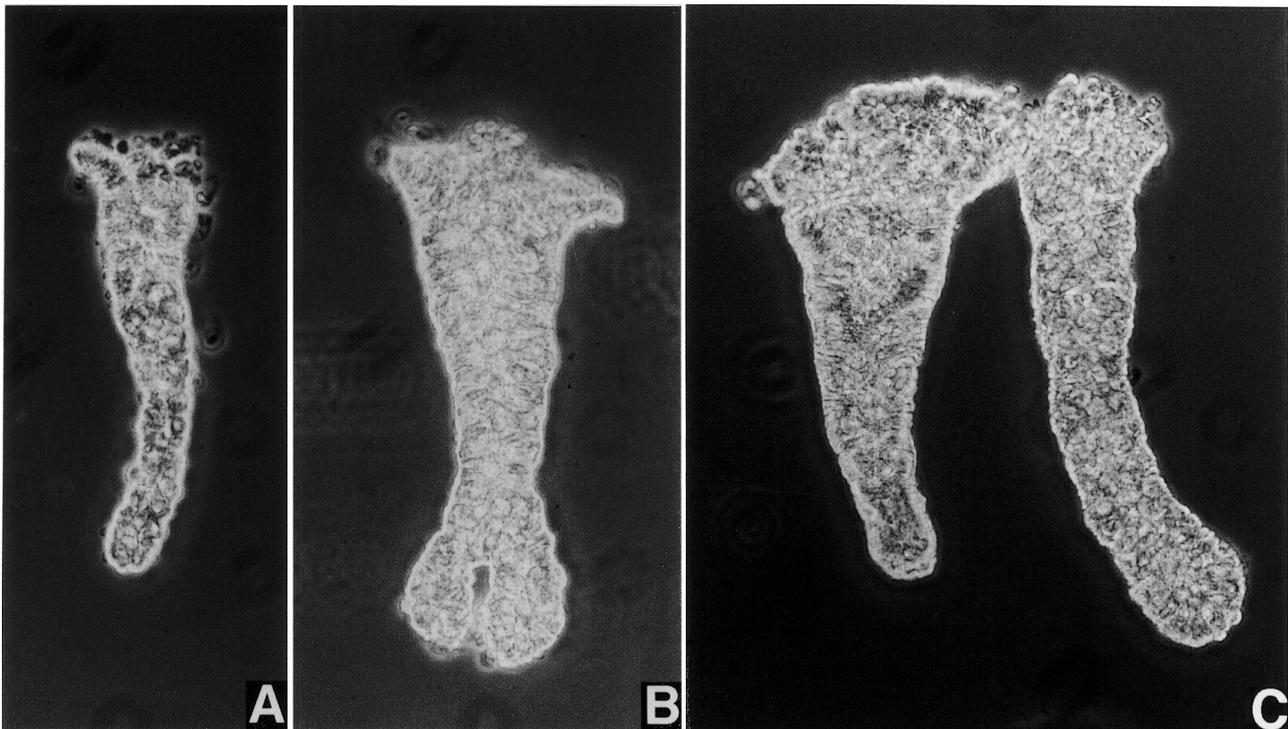


Fig. 2. Application of the crypt isolation technique. A normal crypt (A), an ACF with one bifurcating crypt classified as "B1" (B) and an ACF comprising two "S"-subtype crypts classified as "S2" (C).

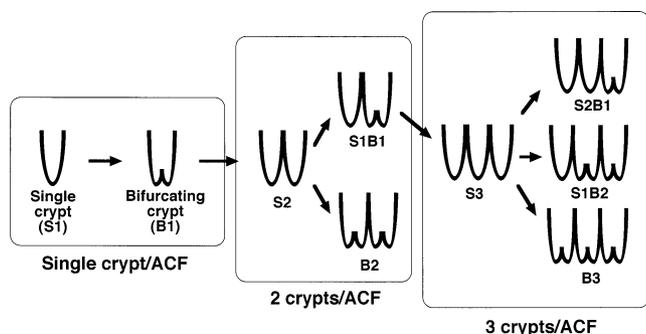


Fig. 3. Development of ACF by fission.

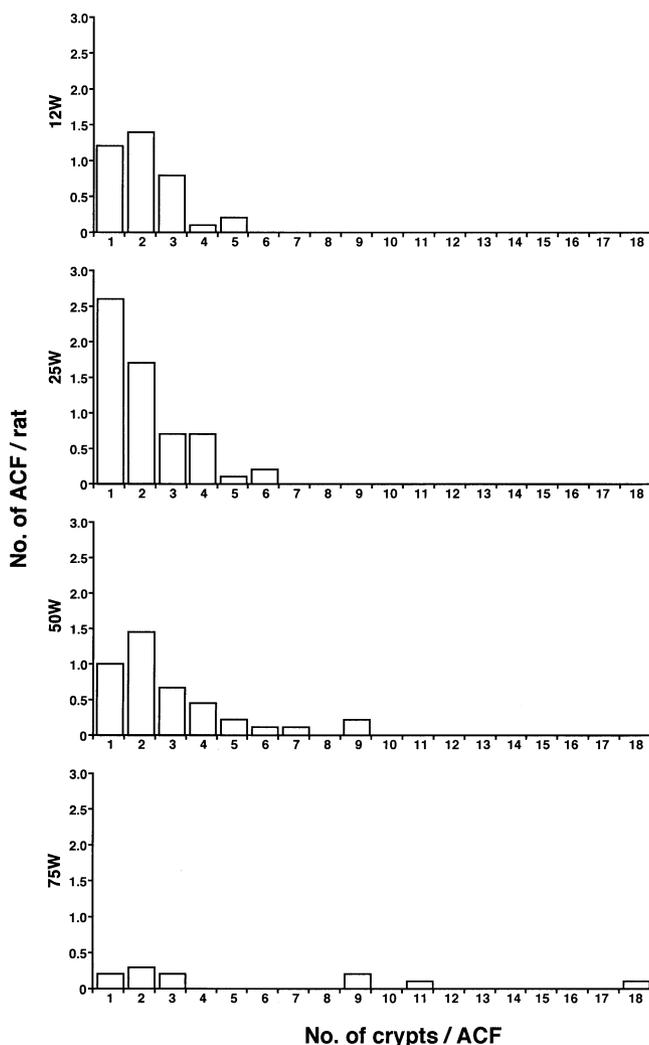


Fig. 4. Data for ACF with different crypt numbers at weeks 12, 25, 50, and 75. The maximum crypt number was 5 at week 12, 9 at week 50 and 18 at week 75. The mean crypt numbers/ACF $\pm$ SD were 2.11 $\pm$ 1.08, 2.10 $\pm$ 1.31, 2.90 $\pm$ 2.06, and 5.55 $\pm$ 5.48 at weeks 12, 25, 50, and 75, respectively. A significant difference ( $P<0.05$ ) between weeks 25 and 50 was observed using Mann-Whitney  $U$ -test.

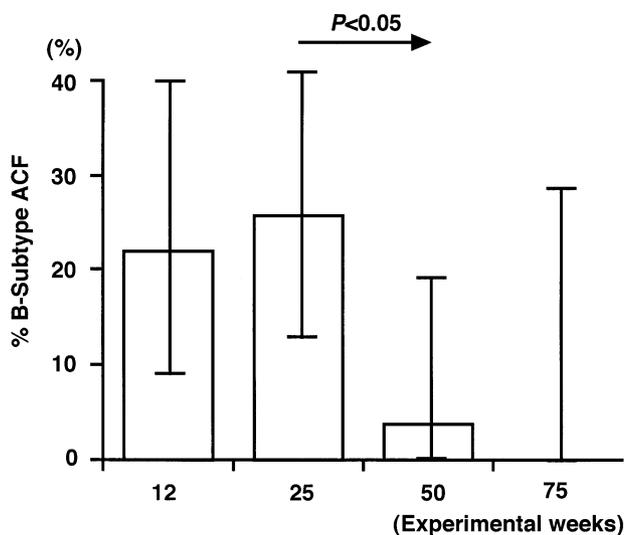


Fig. 5. Percentages of B-subtype ACF. The vertical bar shows the 95% confidence interval (exact method). The Fisher's exact probability test showed a significant decrease ( $P<0.05$ ) from weeks 25 to 50.

**Crypt isolation** The rectum and descending, transverse, and ascending colon were equally subdivided to give segments 1 through 4. The ampulla of the colon and the cecum were classified as segment 5.<sup>18)</sup> The large intestines including the rectum through the cecum (i.e., segments 1 through 5) were excised, stained with 0.2% methylene blue in phosphate-buffered saline, and examined under a stereoscopic microscope. The localization of ACF and the numbers of constituent crypts were recorded. Then the epithelium was treated with 30 mM ethylenediaminetetraacetic acid (EDTA) in Hanks' balanced salt solution and the ACF were isolated from surrounding normal crypts with microforceps under a stereoscopic microscope by using a modification of the method described elsewhere.<sup>16,17)</sup> The isolated crypts were classified into 2 groups, "S" with single or non-bifurcating crypts and "B" with bifurcating crypts (showing a V-shaped cleft at the bottom). ACF containing at least one bifurcating crypt(s) were designated as B-subtype ACF.

**Histological analysis of tumors** Tumors at each location were excised, fixed in 10% buffered formalin, stained with hematoxylin and eosin, and examined under a microscope.

**Statistical analysis** The numbers of crypts in ACF at each time point were compared statistically with the Mann-Whitney  $U$ -test.<sup>19)</sup> The proportions of the bifurcation subtype at each week were presented with 95% confidence intervals obtained by the exact method,<sup>19)</sup> and compared using Fisher's exact probability method<sup>19)</sup> as described earlier.<sup>19)</sup> A ridit analysis was performed for the distributions of ACF and tumors.<sup>20)</sup>

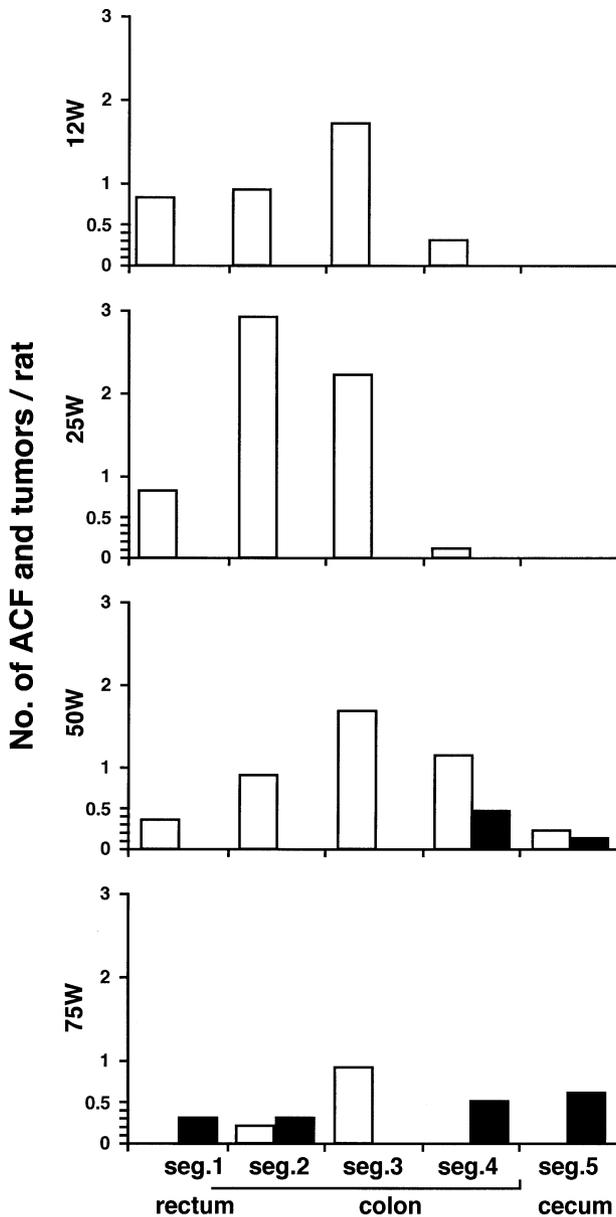


Fig. 6. The distribution of ACF and tumors divided into 5 segments as described in "Materials and Methods" along the large intestine. Note the significance ( $P < 0.000005$ ) from the distal (segment 1 side) to the proximal (segment 5 side) location of ACF from weeks 25 to 50. Tumors were localized in segments 4 and 5 at week 50 and mainly in the same segments at week 75. □ No. of ACF/rat, ■ No. of tumors/rat.

## RESULTS

**Development of ACF** Methylene blue staining of colon epithelium revealed ACF, as shown in Fig. 1, A and B. Isolated ACF crypts (Fig. 2, B and C) were wider and

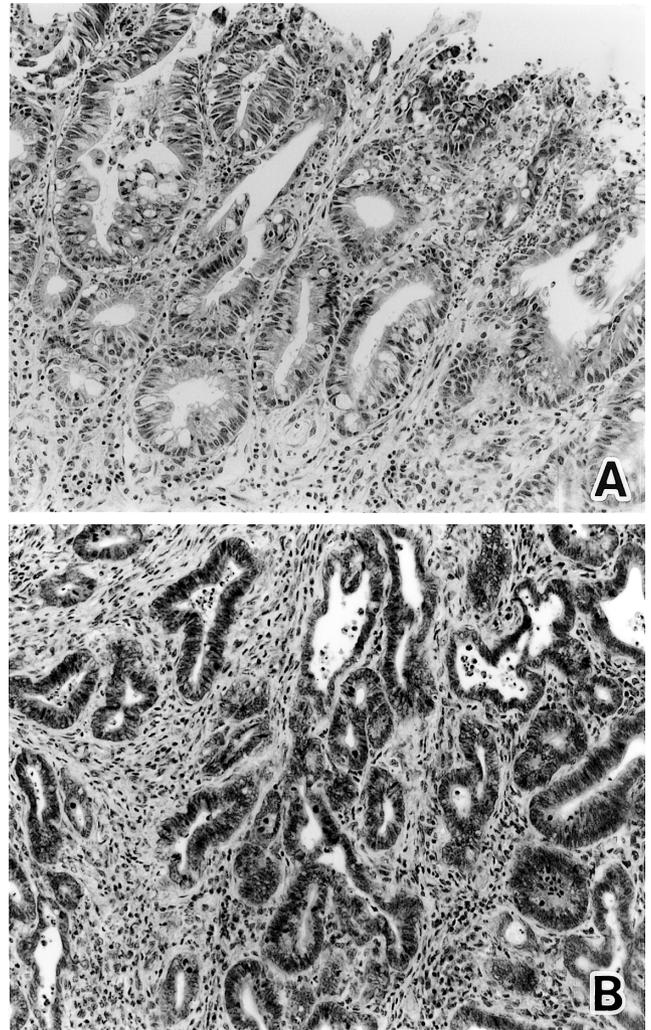


Fig. 7. Tumors developing at weeks 50 and 75. Representative histological appearance of an adenoma (A) and an adenocarcinoma (B).

longer than a normal crypt (Fig. 2A). Classification into non-bifurcating (S-subtype) and bifurcating crypts with a V-shaped cleft (B-subtype) was performed and the numbers of the two types within an individual ACF were counted, as illustrated in Fig. 3.

**Sequential change in crypt numbers in ACF** Data for ACF with different crypt numbers at weeks 12, 25, 50, and 75 are plotted in Fig. 4. The maximum crypt number increased with time and a significant difference ( $P < 0.05$ ) between weeks 25 and 50 was observed using the Mann-Whitney *U*-test.

**Sequential change in percentage of B-subtype ACF** Data for the prevalence of B-subtype ACF are summarized in Fig. 5. At weeks 12 and 25, we found that 7 out of 32

ACF (22%) and 11 out of 43 (26%) were ACF containing B-subtype crypts. At week 50, the rate had decreased dramatically to only 1 out of 27 (3.7%) and at week 75, no B-subtype crypts were found in 11 ACF (see Fig. 5). Fisher's exact probability test showed a significant difference ( $P < 0.05$ ) between weeks 25 and 50.

**Distribution of ACF and colon tumors** The large intestine was divided into 5 segments as described in "Materials and Methods" (Fig. 6). At weeks 12 and 25, ACF were distributed mainly in segments 1, 2, and 3 (88.9% and 98.3%, respectively) and no ACF were found in segment 5, whereas at week 50, the incidences of ACF were highest in segments 3 to 5. This shift in distribution pattern proved to be significant using ridit analysis from weeks 25 to 50<sup>20</sup> (Fig. 6). Tumors included 7 adenomas (Fig. 7A) and 12 adenocarcinomas (Fig. 7B). Some of the latter showed submucosal invasion. They were localized only in segments 4 and 5 at week 50. Later, at week 75, tumors also appeared in segments 1 and 2 (Fig. 6). The distribution patterns at weeks 50 and 75 were not significantly different according to ridit analysis, indicating a preferential distribution in the proximal region.

## DISCUSSION

The present study provided evidence that PhIP induces a heterogeneous population of ACF, some of which are histogenetically involved in the development of colon tumors in the rat. While Thompson *et al.*<sup>3</sup> assayed *S. typhimurium* revertants in the presence of PhIP and found relatively low mutagenicity, Ito *et al.*<sup>5</sup> observed carcinogenicity in the mammary gland and colon in a rat model. Takahashi *et al.*<sup>4</sup> earlier performed a short-term assay of ACF induction with PhIP in rat colon for up to 16 weeks. However, this is the first report of the long-term effect of PhIP on emergence of ACF.

The time-dependent existence of S- and B-subtypes of crypts in ACF suggested that the PhIP-induced ACF developed via the fission mechanism, like normal crypts,<sup>21, 22</sup> but our data indicate that this activity had almost ceased at week 50.

ACF have been reported to be mainly localized in the distal half of the large intestine in rodents treated with carcinogens, including AOM<sup>9</sup> and PhIP.<sup>4</sup> As regards adenocarcinomas, 63% of AOM-induced tumors were found in the distal half of the colon in one experiment in rats.<sup>12</sup> However, in humans, more than half arise in the proximal colon<sup>14</sup> and increasing numbers were reported in the cecal<sup>23</sup> and ascending colon.<sup>24</sup> To explain the discrepancy between animal models and the human cases, our system using PhIP appears to have major advantages. Our long-

term analysis showed that the distribution of the ACF shifted from distal to proximal for our PhIP-induced ACF (Fig. 6) and that tumors were localized only in segments 4 and 5 at week 50. The distribution pattern of the tumors was not significantly changed at week 75 according to ridit analysis, although tumors also appeared in segments 1 and 2. These results suggested that ACF in different segments progress in different manners. Many early-stage ACF might not give rise to adenomas or adenocarcinomas, but rather may disappear, possibly due to apoptosis as a result of over-accumulation of DNA mutation or reversion to normal crypts. The biological fate of other ACF, which tend to be localized proximally and to develop later, might be of greater significance for neoplasia. Several authors<sup>15, 25</sup> have reported poor correlations between the incidence of cancer and the number and size of ACF, but this could be partly explained by heterogeneity of ACF, as shown here, whatever the case. Considering the distribution of large bowel tumors in human patients, PhIP could be a candidate carcinogen responsible to some extent for the recent increase of proximal colon cancers.

On the basis of the present results we propose a classification of ACF according to the presence of bifurcating crypts: (1) proliferating ACF containing bifurcating crypt(s) found mostly in early stages, which may have potential to progress to larger ACF which may proliferate further to become tumors or alternatively may form quiescent/senescent ACF, and (2) quiescent or senescent ACF consisting of only single crypts. Some of those ACF might subsequently be able to enter the proliferation cycle again and develop bifurcations. Others could be senescent and disappear via mechanisms such as apoptosis. Confirmation that this classification is correct will depend on analysis of gene alterations and expression to trace the progression from ACF through adenomas and eventually to adenocarcinomas, or to follow the reversion of ACF to normal crypts or their disappearance. For this purpose, our crypt isolation technique is a powerful method for isolation of DNA without contamination from surrounding normal cells. It clearly warrants further application in molecular analyses of ACF.

## ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare, by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, and by a grant from the Society for Promotion of Pathology of Aichi, Japan.

(Received March 25, 1999/Revised May 11, 1999/Accepted May 13, 1999)

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