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Relationship between the Nature of Mucus and Crypt Multiplicity in Aberrant Crypt Foci in the Rat Colon

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Aberrant crypt foci (ACF) induced in the distal colon of F344 male rats, 4, 8, 12 and 35 weeks after the first administration of 1, 2-dimethylhydrazine-2HCl (DMH) were examined to determine whether a correlation exists between the nature of goblet cell mucin and the number of crypts (crypt multiplicity) comprising the ACF. According to the ACF score calculated from the results of the qualitative observation of sulfomucins (SuMs) and sialomucins (SiMs), the ACF in the 4th week showed a weak correlation between the nature of the mucus and crypt multiplicity, and the ACF of each class showed similar mucous profiles. From the 8th week, a significant difference ($P<0.01$) was recognized between the ACF consisting of 3 crypts or less and those consisting of 4 crypts or more. The proportion of crypts with SiM predominance showed a decrease in the 8th week in the ACF consisting of 1 crypt and in the 12th week in the ACF consisting of 2 or 3 crypts, implying a recovery tendency. The ACF consisting of more than 4 crypts showed little change over time, retaining the tendency of SiM predominance. *Ulex europaeus* agglutinin-I (UEA-I) lectin-positive crypts appeared in the ACF. This finding was significantly more prominent ($P<0.001$) in the ACF with SiM predominance than in the ACF with SuM predominance at each experimental period, and in the 12th week after the first administration of DMH, the incidence of ACF with UEA-I-reactive mucin was decreased in the ACF groups consisting of 3 crypts or less, compared with the ACF groups consisting of 4 or more crypts. These results suggest that the biological quality of mucus in ACF consisting of 4 or more crypts is different from that in ACF consisting of 3 crypts or less. This difference should be considered when ACF are used as an intermediate biomarker of colon cancer.

Key words: Aberrant crypt foci — Rat — Mucus — *Ulex europaeus* agglutinin I — Crypt multiplicity

Aberrant crypt foci (ACF) were reported by Bird[1] to be a "putative preneoplastic lesion" in colon cancer. Later, the incidence of ACF was shown to be modified by carcinogenic accelerators and inhibitors,[2–7] and ACF are now widely considered as an intermediate biomarker of colon cancer. Regarding the biological nature of the crypts of which these ACF consist, a decrease of cellular *N*-acetyl-β-D-glucosaminidase (hexosaminidase) or α-naphthyl butyrate esterase, and an increase of γ-glutamyl transpeptidase in the interstitium have been reported.[8] Also, Sandforth *et al.* reported an increase of sialomucins (SiMs) and a loss of sulfomucins (SuMs) in the goblet cell mucin of ACF.[9] The increase of SiMs, which is a relative increase that results from a decrease of SuMs, has been confirmed in human and rat colon cancer,[9–13] and this observation holds interest beyond the relationship between ACF and colon cancer. The SiM predominance phenomenon in distal colon cancer is considered an oncofetal character which reflects the nature of mucin in the fetal life of human and rats,[14] and this predominance has been shown to correlate with the level of histological malignancy (dysplasia).[15] As one of the structural alterations of mucin, *Ulex europaeus* agglutinin-I (UEA-I)-binding antigenic carbohydrates have been demonstrated in human and rat colon cancer.[16–20] A relationship between the incidence of ACF consisting of 4 or more crypts and carcinogenic frequency has been recognized, and thus the number of ACF consisting of 4 or more crypts is reported to be useful as a parameter to predict the occurrence of colon cancer.[21] Few reports have examined the correlation between the biological nature of the mucus in the crypts and the number of crypts (crypt multiplicity) in ACF. The purpose of this study was to elucidate whether a correlation exists between the biological qualities of goblet cell mucin and crypt multiplicity in ACF using high-iron diamine Alcian blue (HID-AB) and UEA-I lectin histochemical techniques.

MATERIALS AND METHODS

Animals and study design

Twenty 6-week-old male F344: Du Crj rats supplied by Charles River Japan (Kanagawa) were used as the subjects. They were kept in an animal room adjusted to 20–26°C and 40–60% humidity, with a 12-hour light-dark cycle, housed in wire-mesh suspended cages (W260×D380×H180 mm), in groups of 5 rats each, and fed MF solid feed (supplied by Oriental Yeast Co., Tokyo) and water *ad libitum*. ACF were induced by administering a

20 mg/10 ml/kg dosage of 1,2-dimethylhydrazine-2HCl (DMH) twice with a one-week interval by injection into the abdominal cavity.

In the 4th, 8th, 12th and 35th weeks after the first administration of DMH, rats from each group were killed by cutting the abdominal aorta under pentobarbital sodium (Nembutal) anesthetic, and in each instance the colon was extracted and fixed in 10% buffered formalin.

ACF induced in the distal colon (the rectal half of the colon, excluding the plicae part (proximal colon) and a 2-cm portion at the anal end (rectum)) were observed with a stereoscopic microscope after the colon tissue had been stained with 0.2% methylene blue.[1)] ACF in each class, defined in terms of the number of crypts of which they consisted, were counted.

Histological evaluation Each ACF was trimmed to a size of approximately 3×5 mm out of the distal colon, depending on the number of crypts, and embedded in paraffin using routine methods so that the crypts appeared in cross-section in the sectioned specimens. Histological observations were made following hematoxylin-eosin staining and HID-AB staining for the visualization of both SiMs and SuMs.[22)] The lectin immunoperoxidase staining was performed with an UEA-I (E-Y Laboratories, San Mateo, CA)-anti-UEA-I antibody (E-Y) technique, with transverse serial sections of the crypts. Based on these observations the ACF were classified according to crypt multiplicity into groups of ACF consisting of 1, 2, 3, 4, 5 to 7, and 8 or more crypts (HID-AB staining), or 3 crypts or less or 4 or more crypts (UEA-I). Furthermore, the nature of the mucus of each crypt was classified according to the following 5-stage crypt score based on the qualitative ratio of SuM and SiM in the goblet cell mucin. The average crypt score (ACF score) was then calculated for each ACF crypt multiplicity group as described below, and the resulting scores were compared.

For the crypt scores refer to the following

0 points: crypt consisting of only goblet cells with SuM predominance,

1 point: crypt consisting of more goblet cells with SuM predominance than those with SiM predominance,

2 points: crypt consisting of approximately the same number of goblet cells with SuM predominance as those with SiM predominance,

3 points: crypt consisting of more goblet cells with SiM predominance than those with SuM predominance,

4 points: crypt consisting of only goblet cells with SiM predominance.

To examine the change over time of the qualitative ratio of SuM and SiM in relation to crypt multiplicity, the mucin of each ACF group was classified each week for all of the subjects using the above-mentioned crypt score into those with SuM predominance (crypt score: 0 or 1 point), those with near equality of SuM and SiM (crypt score: 2 points), and those with SiM predominance (crypt score: 3 or 4 points), and the incidence frequencies of crypts with SiM predominance were compared.

$$\text{ACF score} = \frac{\left\{\sum\left(\text{crypt score} \times \text{No. of crypts showing this crypt score}\right)\right\}}{\text{No. of crypts in the focus}}$$

The assessment of staining for UEA-I was as follows +, positive goblet cells more than one third per crypt, −, very slight or negative goblet cell mucin, ±, intermediate staining between + and −. Co-incubation using UEA-I that had been absorbed previously with 0.2 *M* α-fucose (Sigma Chemical Co., St. Louis, MO) confirmed the labelling specificity of UEA-I lectin.

Statistical analysis Comparisons between groups in the case of the ACF score in relation to crypt multiplicity were made using the Dunnett multiple comparison procedure. The change over time of crypt incidence frequency, including the incidence of those having SiM predominance, as indicated by the crypt score and the incidence of UEA-I-binding crypts were analyzed using the χ^2 test.

RESULTS

A total of 3 colon tumors was recognized in 2 rats in the 35th week after the first administration of DMH. They were located in the proximal, mid- and distal-colon, respectively, and histopathologically all of them were found to be adenocarcinoma.

Table I shows the results concerning the ACF score in relation to crypt multiplicity after the first administration of DMH. An increase in the mean value of the ACF score was found in the 4th week accompanied by an increase in crypt multiplicity, but the change was not significant. In the 8th week, significant changes were recognized ($P < 0.05$–0.01) in the ACF group consisting of 3 crypts or less, compared with the ACF groups consisting of 4 or more crypts. No significant changes were found among the ACF groups consisting of 4 or more crypts. In the 12th week, significant changes ($P < 0.05$–0.01) were recognized in all of the ACF groups consisting of 3 crypts or less compared with the ACF groups consisting of 4 or more crypts. In the 35th week, the findings showed a tendency similar to that in the 12th week.

Fig. 1 shows the pattern of change in the nature of mucus in the ACF crypts. In the cases of ACF consisting of 1 crypt, while approximately half of the crypts showed SiM predominance in the 4th week, the ratio declined significantly ($P < 0.05$) in the 8th and 12th weeks. In the 35th week, the ratio of crypts with SiM predominance recovered slightly, but this change was not significant. In the cases of ACF consisting of 2 crypts, the ratio of

Table I. ACF Score Based on HID-AB Staining Property of ACF

Times[a] (weeks)	No. of crypts per focus						
	1	2	3	4	5–7	≧8	≧4
4	(63) 2.4±1.2	(54) 2.7±1.0	(29) 2.9±1.0	(5) 3.1±1.0	NA	NA	(5) 3.1±1.0
8	(22) 1.2±1.4	(42) 2.1±1.4[b]	(41) 2.7±1.1[c,d]	(38) 3.2±0.8[c,e]	(16) 3.2±0.6[c,e]	NA	(54) 3.2±0.7[c,e,g]
12	(21) 0.9±1.3	(40) 1.4±1.3	(43) 1.9±1.0[c,d]	(40) 2.7±0.7[c,e,g]	(64) 2.9±1.5[c,e,g]	(9) 2.7±0.6[c,e,f]	(113) 2.8±0.6[c,e,g]
35	(34) 1.8±1.3	(66) 1.2±1.1[b]	(89) 2.2±1.1[e]	(91) 2.8±0.8[c,e,g]	(15) 2.9±0.8[c,e,g]	(31) 2.6±0.2[c,e]	(237) 2.8±0.8[c,e,g]

a) Times after first administration of DMH.
( ): No. of ACF examined.
Values are expressed as mean±SD.
NA: Not applicable.
b, c) Significantly different at $P<0.05$, $P<0.01$, respectively, from the 1-crypt ACF (Dunnett's test).
d, e) Significantly different at $P<0.05$, $P<0.01$, respectively, from the 2-crypt ACF (Dunnett's test).
f, g) Significantly different at $P<0.05$, $P<0.01$, respectively, from the 3-crypt ACF (Dunnett's test).

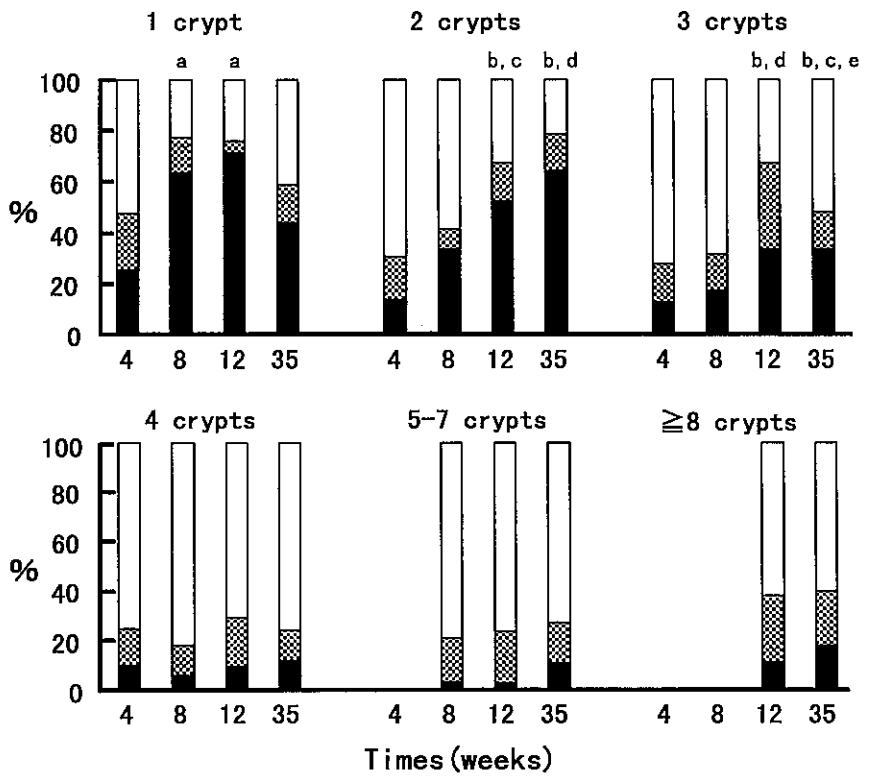

Fig. 1. Mucinous pattern of ACF crypts after the administration of DMH. ■, SuM predominance; ▨, near equality of SuM and SiM; □, SiM predominance. Significantly different from 4th week ($P<0.05^a$, 0.001^b), 8th week ($P<0.01^c$, 0.001^d), 12th week ($P<0.001^e$).

crypts with SiM predominance was found to decline over time. This decline from the 12th to 35th week was significant ($P<0.05$–0.001) compared with that from the 4th to 8th week. No significant change was observed between the 12th and 35th weeks. In the cases of ACF consisting of 3 crypts, while the ratio of crypts with SiM

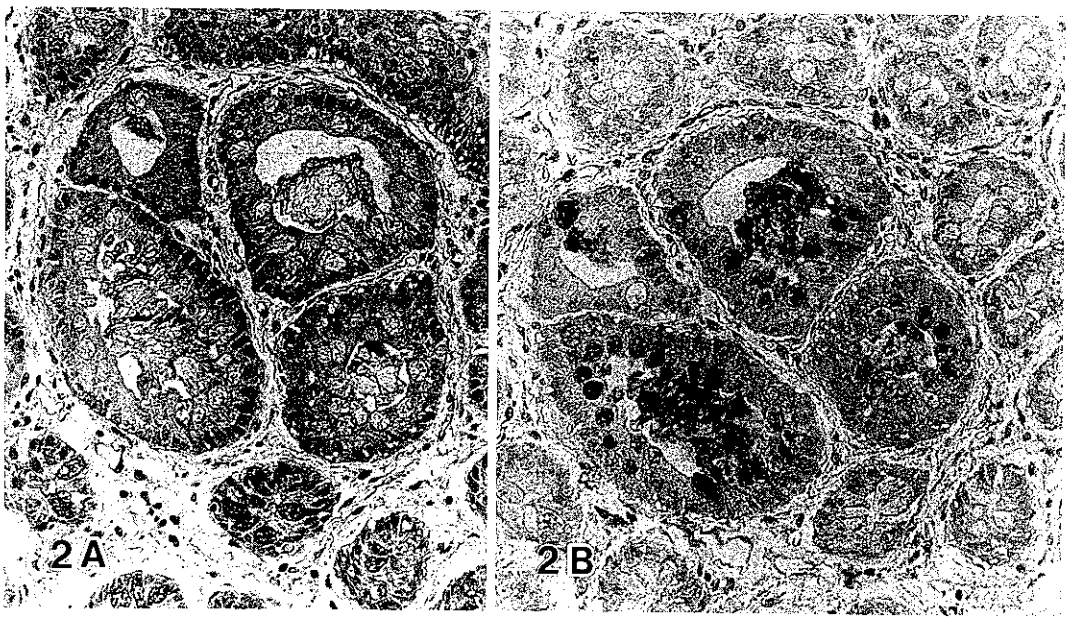

Fig. 2. ACF from a rat at 8 weeks after the first administration of DMH. A, The goblet cell mucin with SiM predominance of ACF are pale in comparison with that with SuM predominance, which appears black in the photograph, of the surrounding normal crypts (HID-AB, ×240). B, An area similar to that shown in Fig. 2A. UEA-I lectin-positivity is seen strongly in the goblet cell mucin of ACF (lectin immunostaining, ×240).

predominance showed almost the same tendency as that in the ACF consisting of 2 crypts up to the 12th week, in the 35th week a significant increase ($P<0.001$) of crypts with SiM predominance was observed compared with the 12th week. The ratio of crypts with SuM predominance showed no change. In the ACF consisting of 4 or more crypts, no change over time was found in the ratio of crypts with SiM predominance. The nature of the mucus in the ACF crypts in the 12th and 35th weeks shared a similar pattern. While the ACF consisting of 3 crypts or less showed a decline in the ratio of SiM-producing goblet cells up to the 12th week after the first administration of DMH, those consisting of 4 or more crypts showed no change in the ratio of SiM-producing goblet cells in any sampling week, thus indicating an apparent difference in the nature of the mucus between the ACF consisting of 3 crypts or less and those consisting of 4 or more crypts.

The goblet cell mucin in cross-sections of normal distal colons was hardly stained with UEA-I lectin. However, the surface coats and the goblet cell mucin of the adenocarcinoma on the distal colon mentioned above were strongly stained with UEA-I lectin. The goblet cell mucin and crypt secretory products were stained with UEA-I lectin on some ACF, and not stained on other ACF (Figs. 2 and 3).

The relationship between ACF score by HID-AB staining and UEA-I lectin staining is shown in Fig.4. The incidence of UEA-I lectin-positive ACF was 18.9–34.4% of the SuM-dominant ACF (except for 4.6% in the 12th week). It was 77.8–71.5% of the SiM-dominant ACF (except for 98.9% in the 8th week). The positive reaction rate of the SiM-dominant ACF was significantly higher than that of the SuM-dominant ACF ($P<0.001$). The incidence of strongly stained crypts was also higher among the SiM-dominant ACF than among the SuM-dominant ACF. After the first DMH administration, the number of UEA-I lectin-positive crypts in the SiM-dominant ACF increased significantly in the 8th week ($P<0.001$), and then decreased to the same level as the 4th week. However, many ACF were strongly stained again in the 35th week ($P<0.001$). The most ACF were positive in the 8th week.

The relationship between the number of crypts and the incidence of UEA-I lectin-positive crypts is shown in Fig. 5. The SiM-dominant ACF with a high UEA-I lectin-positive rate were divided into two groups, one of ACF consisting of 4 crypts or more, whose relation with colon cancer has been indicated, and the other of ACF consisting of 3 crypts or less. The ACF consisting of 3 crypts or less were significantly different from the ACF consisting of 4 crypts or more in the degree of staining in the 4th

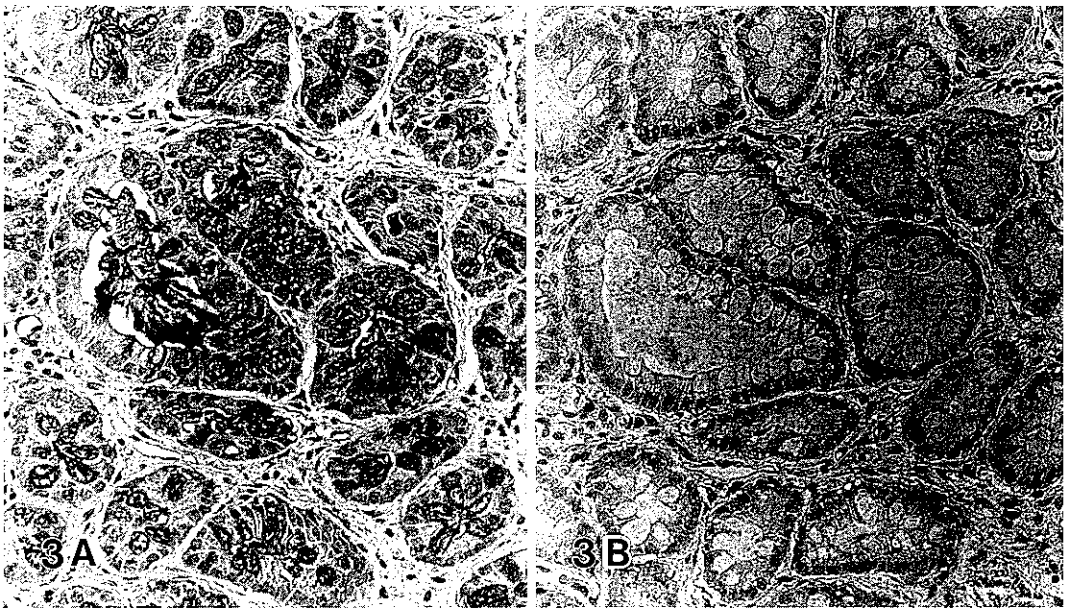

Fig. 3. ACF from a rat at 8 weeks after the first administration of DMH. A, The goblet cell mucin with SuM predominance of ACF is seen as black, similar to that of the surrounding normal crypts (HID-AB, ×240). B, An area similar to that shown in Fig. 3A. UEA-I lectin-positivity is not seen in the goblet cell mucin of ACF (lectin immunostaining, ×240).

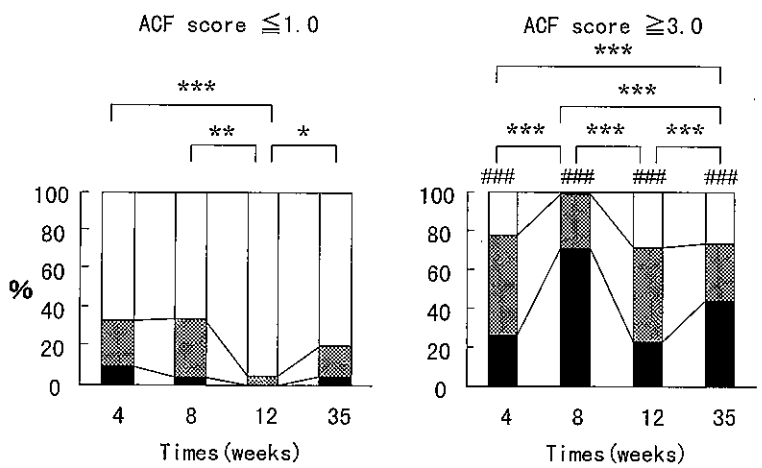

Fig. 4. Relationship between ACF score based on HID-AB staining property and incidence of UEA-I lectin-positive crypts in ACF. □, very slight or negative goblet cell mucin; ▨, intermediate staining; ■, positive goblet cells amounting to more than one-third per crypt. Statistical significance was analyzed using the χ^2 test. ###: Significantly different from the ≦1.0 ACF score at $P<0.001$. *, **, ***: Significantly different at $P<0.05$, 0.01, 0.001, respectively.

week ($P<0.01$), while they were not significantly different in the 8th week. However, the incidence of positive crypts among the ACF consisting of 4 crypts or more was significantly higher than that of the ACF consisting of 3 crypts or less after the 12th week ($P<0.001$). A reduction of staining on the latter was observed.

DISCUSSION

In the present study, the proportion of crypts with SiM predominance was decreased, and the proportion of crypts with SuM predominance was increased in the ACF consisting of 1 to 3 crypts in the 8th and 12th weeks

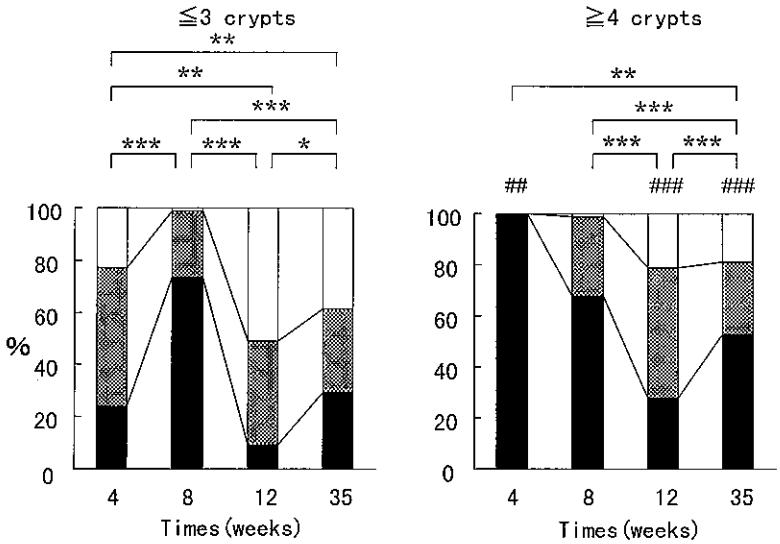

Fig. 5. Relationship between crypt multiplicity and incidence of UEA-I lectin-positive crypts in ACF. □, very slight; ⊠, intermediate staining; ■, positive goblet cells amounting to more than one-third per crypt. Statistical significance of differences was analyzed using the χ^2 test. ##, ###: Significantly different from the ≦3 crypts at $P<0.01$, 0.001, respectively. *, **, ***: Significantly different at $P<0.05$, 0.01, 0.001, respectively.

compared with those observed in the 4th week. In the ACF consisting of 4 or more crypts, little change was recognized after the 4th week. From these results, in the 4th week following the first administration of DMH there seems to be no significant correlation between the nature of mucin in the ACF and crypt multiplicity. All ACF appear to have had a similar mucous profile at this time. However, later, in the ACF consisting of 3 crypts or less, the proportion of crypts with SuM predominance was found to increase, thus implying a recovery tendency. The incidence of UEA-I lectin-positive crypts of SiM-dominant ACF indicated the same tendency. The incidence after the 12th week was significantly higher among the ACF consisting of 4 crypts or more than among those consisting of 3 crypts or less. This finding indicates the recovery of the biological disposition of the ACF consisting of 3 crypts or less. Caderni *et al.*[23] recognized a decrease of SuM and an increase of SiM accompanied by an increase in the number of crypts comprising the ACF, in unsectioned rat colons stained with HID-AB after the administration of a carcinogenic substance. They also found a borderline-significant association at $P=0.057$ between the presence of a tumor and SiM-producing ACF. In the ACF consisting of 4 or more crypts in the present study, little change occurred over time and the tendency towards SiM predominance remained. As mentioned earlier, the increase of SiM and decrease or loss of SuM were observed in the colon cancer and ACF of humans and rats,[9-13] and this tendency has been shown to correlate with the level of histological malignancy (dysplasia).[15] Increased binding of UEA-I lectin has frequently been observed in highly dysplastic adenoma and cancer.[24] UEA-I lectin could be a useful tumor marker in the distal colon.[17,20] In the present study, the incidence of UEA-I lectin-positive crypts was higher in the SiM-dominant ACF than in the SuM-dominant ACF, indicating that the end of the glycochain could change in ACF as well as in colon cancer. This is an interesting finding regarding the relation of ACF and colon cancer. The difference of UEA-I lectin staining between dysplastic ducts of ulcerative colitis and non-dysplastic regenerating epithelium has also been reported.[25]

Counts of the number of ACF have been done in studies searching for indicators of colon cancer, and an important problem is the identification of the optimal time at which the count should be done. Pretlow *et al.*[21] have recognized a similar tendency in the development frequency of tumors induced by the administration of carcinogenic substances and the incidence of ACF consisting of 4 or more crypts. Their data showed almost the same incidence of such ACF in the 12th and 36th weeks after administration, thus raising the possibility that the 12th week is the appropriate time for the evaluation. Our data showed, with regard to the nature of mucus in the ACF, that almost the same ACF scores were obtained in the 12th and 35th weeks in groups classified according to crypt multiplicity. These results imply that no change in the nature of the mucus occurs, though the total number of ACF shows some changes after the 12th week. Count-

ing the number of ACF consisting of 4 or more crypts after the 12th week appears to be useful in the search for indicators of colon cancer. Kristiansen[26] also reported that the detection of a high number of ACF with low crypt multiplicity (1–3 AC/focus) in the mouse colon after two heterocyclic amine treatments was not indicative for the endpoint colon cancer.

In conclusion, we found that the biological difference between the ACF consisting of 4 crypts or more and the ACF consisting of 3 crypts or less was reflected by mucinous disposition and crypt multiplicity (which are common to colon cancer), in DMH-induced ACF in rat distal colon. These results support the theory of Pretlow *et al.*[21] and Kristiansen[26] that the existence of ACF consisting of 4 crypts or more is important as a risk factor for colon cancer.

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