ABSTRACT

Aberrant crypt foci (ACFs) are the earliest identifiable epithelial lesions thought to precede the development of a subset of colon tumors. To assess their predictive value to adenoma development, we have tested in mice whether the development of ACFs and adenomas is controlled by the same genes. Therefore we used the CcS/Dem series of recombinant congenic strains, in which the effect of multiple susceptibility genes might be studied separately. We investigated susceptibility to ACFs in nine CcS/Dem strains and their parental strains, BALB/cHeA and STS/A, 4 weeks after s.c. injection of 1,2-dimethylhydrazine (20 mg/kg body weight). The nine CcS/Dem strains, the BALB/cHeA strain, and the STS/A strain exhibited different patterns of susceptibility to ACFs and adenomas, demonstrating that different subsets of susceptibility genes are involved. Therefore, in evaluating the role of ACFs as a predictive marker for adenoma development, genetic factors must be taken into account.

INTRODUCTION

Colon tumorigenesis is a complex, multistep process in which various stages can be distinguished. Genetic as well as nutritional factors play an important role during the development of colon tumors (1).

ACFs (1) are early changes in the colonic mucosa of animals treated with carcinogens (2–6) and the grossly normal colonic mucosa from patients with colon cancer (7), suggesting that ACFs are lesions that are associated with colon tumorigenesis. They are thought to be the earliest recognizable precursors of colon adenomas (4, 5, 7, 8). ACFs as well as colon adenomas in man contain mutations of KRAS and APC (9, 10), two genes important in colon tumorigenesis (11), supporting the role of ACFs as precursors of a subset of colon tumors.

In mice, ACFs (3), as well as colon adenomas (12), can be induced by the carcinogen DMH (3). Several inbred strains and substrains of mice differ widely in susceptibility to DMH-induced colon tumors (13, 14), due to multiple, nonlinked genes (15). In this study, to determine whether the number of ACFs is predictive of colon tumor development, we investigated the genetic control of the development of ACFs and compared the susceptibility to ACFs with the susceptibility to colon adenomas.

We used the CcS/Dem series of 20 homozygous recombinant congenic strains of mice, derived from the inbred strains BALB/cHeA (relatively resistant to DMH-induced colon adenomas) and STS/A (susceptible; Refs. 16 and 17). Each CcS/Dem strain contains a random subset of about 12.5% genes from the STS/A strain, whereas the rest originates from the BALB/cHeA strain (17–20). This led to separation and fixation of the multiple, nonlinked genes involved in the control of colon tumorigenesis in individual CcS/Dem strains (16).

Previously, we determined the differences in colon adenoma susceptibility between the 20 CcS/Dem strains and both the parental strains BALB/cHeA and STS/A after induction with DMH (17). In the present experiments, we studied the susceptibility to ACFs in the BALB/cHeA and STS/A strains and nine CcS/Dem strains after single s.c. injections of DMH (20 mg/kg body weight) and compared their susceptibility to ACFs with their susceptibility to adenomas.

MATERIALS AND METHODS

Animals. Mice 12–15 weeks of age were used. The animals were fed a standard laboratory diet (AM II pellets; Hope Farms, Woerden, the Netherlands) and acidified water (pH 2.5–3) ad libitum in an air-conditioned room with a 12-h light-dark cycle. The BALB/cHeA, STS/A, and CcS/Dem strains were described previously (17–20).

Carcinogen. DMH (Sigma Chemical Co.) was dissolved immediately before use in 1 mM EDTA solution, which was adjusted to pH 6.5 with sodium hydroxide.

Induction of ACF. On average, 8 mice of the CcS/Dem strains CcS-3, -5, -11, -15, -16, -17, -18, -19, and -20 and the two parental strains, BALB/cHeA and STS/A, were injected s.c. with single doses of 20 mg DMH/kg body weight. The mice were sacrificed 4 weeks after DMH treatment. In addition, groups of, on average, eight mice of the strains BALB/cHeA, STS/A, and CcS-19 were killed 2, 8, and 12 weeks after DMH treatment. Colons were removed, slightly distended by filling with 0.9% NaCl solution under mild pressure, slit open longitudinally from anus to cecum, and fixed flat in ethanol-acetic acid formal saline (21). The fixed colons were stained with hematoxylin, and the occurrence and distribution of ACFs were determined using light microscopy (magnification, 10 × 12.5); the number of ACFs per colon, their locations, and the number of aberrant crypts per focus were recorded. To determine the distribution of ACFs, the colon was divided in segments 1 cm long, numbered from the anal sphincter. Using the description of different features of aberrant crypts by Bird (2) and McLellan and Bird (3, 4), we distinguished four different types of ACFs, using as criteria the extent of atypia and the presence or absence of elevated growth. We also distinguished between single and multiple crypt lesions.

Type 1: the crypt has a normal size but shows increased cellularity. Mucinous vacuoles are present in the majority of cells.

Type 2: the crypt has an increased diameter and increased cellularity. The cells are basophilic, and mucinous vacuoles are present in the majority of cells. These lesions are slightly elevated above the normal surrounding mucosa, suggesting an increased amount of stromal tissue. They are covered by normal luminal epithelium.

Type 3: the crypt has an increased diameter and highly increased cellularity, with epithelium exhibiting pseudostratiﬁcation and basophilic cytoplasm. The vacuoles are absent in the most cells (increased atypia). Mitoses can easily be found. These lesions are not elevated.

Type 4: the epithelium is similar to that of type 3, but the crypt is elevated. A lesion of type 2, 3, or 4 may comprise several neighboring or branching crypts. Such lesions were classiﬁed as multiple crypt lesions.

The mucosa in the direct vicinity of Peyer’s patches was not evaluated, because of the normal occurrence of aberrant crypt-like structures in untreated animals.

Induction of Adenomas. The procedure was described in detail previously (17). Briefly, the mice of the two parental strains and 20 CcS/Dem strains were injected weekly, for 26 weeks, with DMH (15 mg/kg body weight, s.c.) and...
sacrificed 32 weeks after the first injection. At autopsy, the numbers, sizes, and locations of colon tumors were recorded using a dissection microscope. Representative samples of colon tumors in all strains were investigated microscopically.

Statistical Analyses. The analyses were based on two-way (strain and sex) and three-way (strain, sex, and week) ANOVA. Numbers of ACFs and adenomas were square-root transformed and power transformed using an exponent of 0.43, respectively, to improve normality and constancy of variance of the residuals.

To simplify the model, nonsignificant \((P > 0.05)\) interaction terms and main effects \((P > 0.10)\) of sex and time were excluded one by one, prior to making conclusions about strain differences. If an interaction between strain and time had to be included in the model (indicating that strain differences might be time specific), strain differences were analyzed at each time point separately. If only the main effect of time remained in the model (indicating that the response variable changed over time equally for all strains), strain differences were evaluated adjusted for time changes \((i.e., \text{averaged over time points})\) using the two-way ANOVA model with main effects only.

When pairwise comparisons of strains were performed, or when comparisons of strains had to be done separately at each time point, \(P\) values were adjusted for multiple comparisons using the Hommel procedure \((22)\).

To evaluate a possible relationship between susceptibility to ACFs and susceptibility to colon adenomas, the Spearman correlation coefficient was calculated after the numbers of ACFs and adenomas were converted to ranks.

RESULTS

Susceptibility to ACFs

Fig. 1A shows the number of ACFs per mouse 4 weeks after DMH treatment in various CcS/Dem strains and the parental strains BALB/cHeA and STS/A. The strain had a highly significant influence on the number of ACFs per mouse \((P = 0.0004)\). There was no evidence that these strain differences might be sex related or that there was an overall difference between males and females.

The strain BALB/cHeA is susceptible to ACF, whereas the strain STS/A is resistant to ACF. The CcS/Dem strains, which differed significantly from BALB/cHeA in numbers of ACFs (CcS-5 and CcS-20), were considered resistant, whereas the CcS/Dem strain, which differed significantly from STS/A (CcS-11), was considered susceptible. Several CcS/Dem strains were intermediate.
GENETIC SUSCEPTIBILITY TO ACF AND ADENOMAS

Fig. 2. Number of aberrant crypts in individual males (O) and females (X) of the strains BALB/cHeA, STS/A, and CcS-19, observed 2, 4, 8, and 12 weeks after single doses of the carcinogen DMH (20 mg/kg body weight, s.c.).

Detailed Analysis of ACFs in BALB/cHeA, STS/A, and CcS-19 Strains

For the strains BALB/cHeA (high number of ACFs), STS/A (low number of ACFs), and CcS-19, we investigated the total number of ACFs per mouse, the percentage of single and multiple crypt lesions, and the location of the different types of ACFs at different time intervals after a single dose of DMH.

Total Number of ACFs and Percentage of Single and Multiple Crypt Lesions. Overall, there was no difference in the total numbers of ACFs and the percentages of single and multiple crypt lesions between males and females (Fig. 2). Comparison of the strains CcS-19, BALB/cHeA, and STS/A on weeks 2, 4, 8, and 12 showed a significant difference between BALB/cHeA and STS/A at all time points, BALB/cHeA having a higher number of ACFs (higher number of single as well as multiple crypt lesions) per mouse than STS/A (P < 0.00001). No difference between CcS-19 and BALB/cHeA was observed. CcS-19 had a higher number of ACFs per mouse than STS/A (P ≤ 0.0024). In addition, the CcS-19 strain had a higher number of single crypt lesions as well as multiple crypt lesions compared with STS/A (P < 0.0001) at all time points.

In general, more single crypt lesions were found (mean frequency, 73.4%) than multiple crypt lesions (mean frequency, 26.6%). The mean frequencies of multiple crypt lesions in BALB/cHeA (29.8%) and CcS-19 (28.8%) strains were higher than in the STS/A strain (13.2%; P < 0.0001). Overall, the total number as well as the frequency of multiple crypt lesions increases from weeks 2 to 8, whereas, it remains stable thereafter (P < 0.001). For the number of single lesions, there is no evidence of a change over time.

Comparison by Type of Lesion. The mean frequencies of the four types of ACFs for all experimental groups pooled were 8.4, 28.2, 29.5, and 33.9%, respectively. For each type, the multiplicity in individual strains and sexes and at different time points followed the tendency exhibited by the total number of ACFs. Although there were some individual cases of significant differences between weeks, they did not indicate any clear trend and, therefore, permit no further conclusion.

Location of ACFs and Adenomas. All adenomas were found in the distal half of the colon (17), where also 75% (or more) of the ACFs were located (Fig. 3).

Susceptibility to ACFs and Adenomas. There is no obvious relationship between susceptibility to ACFs and susceptibility to colon adenomas (Spearman correlation coefficient, 0.26; Fig. 4). For the parental strains BALB/cHeA and STS/A and the strain CcS-5, susceptibility to early lesions (expressed as the number of ACFs) did not correspond with susceptibility to colon adenomas (Fig. 1B); BALB/
CcS mice are resistant to colon adenomas but contain a relatively high number of ACFs, whereas mice of the strains STS/A and CcS-5 are susceptible to colon adenomas (also see legend of Fig. 1B) but contain a low number of ACFs. For the strains BALB/cHeA and STS/A, these discrepancies between susceptibility to early lesions and susceptibility to colon adenomas after DMH treatment were observed at all time intervals when susceptibility to ACFs was tested. However, in some CcS/Dem strains, there seems to be an agreement between the number of ACFs and the number of colon adenomas; CcS-11 is susceptible to adenomas and has a high number of ACFs as well, whereas CcS-20 is resistant both to colon adenomas and ACFs.

DISCUSSION

Aberrant Crypts. Considerable differences were found in the multiplicity of ACFs between the strains tested, indicating that genetic factors are involved. Seventy-five percent (or more) of the ACFs were found in the distal part of the colon, where all adenomas were also located. This location contrasts with the preferential location of ACFs in the central part of the colon, as reported by Carter et al. (23). However, they used multiple injections of DMH; therefore, the two results are not directly comparable. Each type of ACF was found in the strains BALB/cHeA, STS/A, and CcS-19 at all time points. We found no evidence for the transition from one type of ACF to the other. Therefore, we cannot draw any conclusion with respect to which of these types could be the precursor of the adenomatous polyps, although the aberrant crypt types 3 and 4 are likely candidates because of the epithelial atypia and superficial and elevated growth (type 4).

Colon Adenomas. In the previous study (17), strains CcS-4, -5, -7, -11, -16, -17, -18, and -19 were susceptible to DMH-induced colon tumors, whereas strains CcS-6, -8, -10, -15, and -20 were resistant. The tumors in the colon were histologically diagnosed as benign adenomas and some carcinomas in situ. The tumors were sometimes large, showing superficial and polypous (exophytic) growth. Other tumors showed focal atypia and dysplasia (carcinoma in situ). No tumor showed growth beyond the muscularis mucosae; they were not invasive and did not metastasize.

Using the recombinant congenic strain system, we mapped two of the genes responsible for the predisposition to colon tumors (measured as the number of colon adenomas), Sccl (24) and Scc2 (25) to chromosome 2. Sccl has been mapped to a region less than 2.4 cM in the vicinity of D2Mit66, and Scc2 is located centromerically from D2Mit66, near D2Mit7 (25).

Susceptibility to Aberrant Crypts versus Colon Adenomas. No agreement was found between the susceptibility to ACF and colon adenomas in the strains BALB/cHeA, STS/A, and CcS-5. The strain BALB/cHeA has a relatively high number of ACFs but a low number of adenomas. This indicates that an ACF does not necessarily progress to an adenoma. On the other hand, strains CcS-5 and STS/A have low numbers of ACFs but develop high numbers of adenomas. This may be due to a difference in growth or progression rate of the ACFs in strains CcS-5 and STS/A versus the ACFs in strain BALB/cHeA, suggesting that the genes responsible for the discrepancy between the numbers of ACFs and adenomas operate in the transition period between the two stages. A stage-specific influence of oncogenes and tumor suppressor genes has been proposed previously (11). The observed strain differences in the numbers of ACFs may also be due to genetically determined differences in functional characteristics of the normal mouse intestinal epithelium, which influence the propensity for the development of ACFs but not the subsequent development of adenomas. After the genes influencing the number of ACFs have been mapped, this hypothesis can be tested. Alternatively, there may be other precursor stages not recognizable with the methods used here, or the ACFs occurring in the BALB/cHeA strain need not necessarily be functionally identical to those observed in the STS/A strain. This latter possibility is supported by mutation analysis of the genes KRAS and APC in ACFs and polyps in human colons, which suggests that if a RAS gene mutation occurs as the first genetic event, a nondysplastic ACF will form, which has little potential to progress, whereas if an APC mutation occurs first, a dysplastic ACF will result, which has the capacity to progress. This progression is driven by subsequent mutations in RAS and other genes (9). Within this context, it would be interesting to compare Kras and Ape mutations in the different types of ACFs and adenomas in the mouse strains tested here. Finally, because the induction scheme for ACF in this and other studies was quite different from that used for adenomas (2–6, 26), the influence of repeated DMH injections on initiated ACFs and early tumors in different strains should be studied to further elucidate the dynamics of changes in the mucosa, which result in the discrepancy between the numbers of ACFs and adenomas. Previous studies on a limited number of strains suggested a correlation between susceptibility to ACFs and adenomas (26); however, this correlation may be accidental, because our tests of additional strains do not confirm it. Whatever the mechanism, the absence of an agreement between susceptibility to ACFs and colon adenomas in several strains implies that at least some genes that control the susceptibility to ACFs are different from the genes that are involved in the susceptibility to colon adenomas. Therefore, in evaluating the predictive value of the number of ACFs for colon tumorigenesis in different experiments, with different induction protocols, genetic factors must be taken into account.

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