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Comparative Study of Histopathologic Characterization of Azoxymethane-induced Colon Tumors in Three Inbred Rat Strains

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To obtain controlled genetic variation, colon cancer was chemically induced by use of four subcutaneous injections of azoxymethane (15 mg/kg of body weight/wk) to rats of 3 inbred strains (BDIX/OrlIco, F344/NHsd, WAG/Rij). The selection was based on the availability of established colon cancer cell lines arising from these particular strains. In the first experiment, only female rats were used; in the second experiment, both sexes were studied. The goal was to select a rat strain giving the highest tumor frequency with the shortest latency period in reproducible manner. The histologic characteristics should resemble the corresponding human tumors. The size of the tumors should be at about 1 cm in diameter, as these tumor cells were intended to be used in future transplantation studies. The two experiments yielded highly reproducible results: histologic evaluation of all colon tumors in all three rat strains revealed adenomas and adenocarcinomas closely resembling their human counterpart. The BDIX strain had the highest tumor frequency (75%) in both sexes and the shortest minimal latency period (28 weeks in experiment 1; 23 weeks in experiment 2). Tumor size of about 1 cm in diameter was found most often in the BDIX strain. On the basis of results of these two experiments, the BDIX strain has been selected for future study.

Colorectal cancer (CRC) is still one of the most common malignant diseases in the western world. Despite advances in surgical and adjuvant therapy, prognosis remains rather poor. New treatment modalities can be tested in animal models, but in them, spontaneous CRC is a rare event, the incidence in rats being less than one percent. Higher incidence can be obtained by use of chemical induction. Dimethylhydrazine and its metabolite, azoxymethane (AOM), are frequently used to induce CRC in animal models. These compounds must be activated metabolically to become carcinogenic, and most of the activation occurs in the liver (1).

Dimethylhydrazine is oxidized to azomethane, which then forms AOM, which is then hydroxylated to form methylazoxymethanol. In the final step, methylazoxymethanol is converted to formalin and methyldiazoniom ion, which is responsible for alkylation of DNA, RNA, and protein, principally in the colon, liver, and kidneys (2-4). Subcutanous injections of AOM have frequently resulted in high incidences of CRC in rats, but great variation in the results constitutes a problem (5). Chemical carcinogens like AOM not only induce colon tumors, but also have unwanted side effects, such as induction of other tumors (e.g., in the small intestine [5]).

Colorectal cancer incidence is a function of genetic background, exposure to the carcinogen, and time. Exposure to the carcinogen is highly dependent on its bioavailability and bioactivity. To obtain less variable results, more systematic research into the determining factors that induce CRC in experimental models is needed.

Liver metastases do not usually develop in high frequencies in rats with colon carcinomas (6). To model liver metastases, intraportal injection of rat colon cancer cells derived from cell lines is often used (7, 8). A more biological way would be to use a heterogeneous cell population instead of highly selective cell lines to obtain liver metastases for immune therapy studies. This heterogeneous cell population is planned to be derived from chemically induced colon tumors in syngeneic models. To achieve this long-term goal, standardization and reproducibility of the chemical induction of colon tumors are important.

The objective of the study reported here was to induce CRC by AOM injections in three inbred rat strains (BDIX/OrlIco, F344/NHsd, and WAG/RijHsd). The selection was based on the availability of established colon cancer cell lines derived from these inbred strains (9, 10). The further purpose of the study was to find a rat strain that would develop relatively quickly reproducible high incidence of CRC (80 to 100%) with a tumor size of about one centimeter after AOM injections, to be able to obtain a sufficient number of heterogeneous cancer cells for liver metastases model transplantation studies. The histologic characteristics must resemble those of human CRC.

Materials and Methods

Animals. At the time of arrival at the laboratory, the animals

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had a certified health report in accordance with the FELASA recommendations (11). Only F344 and WAG rats were tested for rodent helicobacters, and were found to be negative.

Experiment 1. Female BDIX/OrlIco rats, 12 weeks old, were purchased from IFFA Credo (L'Abresle Cedex, France); female F344/NHsd rats, 9 weeks old, were purchased from Harlan, Oxon, UK; and female WAG/RijHsd rats, 9 weeks old, were purchased from Harlan NL (Harlan, Horst, The Netherlands). All ages refer to the time of the first AOM injection.

Experiment 2. Female and male BDIX/OrlIco rats, 9 weeks old, were purchased from IFFA Credo (L'Abresle); female and male F344/NHsd rats, 8 weeks old, were purchased from Harlan (Oxon, UK); and female and male WAG/RijHsd rats, 8 weeks old, were purchased from Harlan NL (Harlan, Horst, The Netherlands). All ages refer to the time of the first AOM injection.

Animal housing. All experiments were performed in accordance with UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia (12) and had been approved by the Animal Experimentation Inspectorate of the Danish Ministry of Justice. The animals were kept under standard laboratory conditions: room temperature between 20 and 24°C, relative humidity between 50 and 60% and a 12-h light/dark cycle (lights on from 6 AM to 6 PM). Temperature and relative humidity inside the isolator were not recorded separately. The bedding consisted of irradiated aspen wood chips (Tapvei, Oy, Kaavi, Finland), and the cages were changed twice a week, inside and outside the isolator. Outside the isolator, the rats were given aspen wood shavings and/or wooden blocks (Tapvei) twice a week as environmental enrichment. The animals in both experiments were housed in groups of two in Macrolon type-III cages (Scanbur A/S, Køge, Denmark). To protect the personnel and the environment from this carcinogen and its metabolites, the AOM-treated animals had to be housed inside an isolator (Isotec type 13366 (M50), Harlan, The Netherlands) with negative pressure (3 mmH_2O) until two weeks after the last AOM injection. After carcinogen induction, the rats were taken out of the isolator and housed in the room together with the control animals. From this time, all rats had ad libitum access to non-acidified water in water bottles, the contents of which were changed once a week.

For each experiment, the same batch of a pelleted rat chow (Altromin 1324, Chr. Petersen Inc., Denmark) was available ad libitum, inside and outside the isolator. Fresh food was given at least once a week on top of the remaining food. In both experiments, two batches of diet were used. In the first experiment, rat cages, according to strain, were positioned in a vertical row on the shelves, whereas in the second experiment, they were positioned in a horizontal row. Animals inside the isolator and control animals outside the isolator (the control group was housed outside the isolator in the same animal room, as there was no more space available in the isolator) were allowed ad libitum access to acidified tap water (acidified with HCl, pH 3, to reduce bacterial growth) via water bottles (1 L), the contents of which were changed every four weeks during their stay in the isolator (total, eight weeks). If necessary, bottles were filled during this four-week period.

Genetic analysis of the strains. Ten rats per strain of experiment 1 (of the F344 strain only nine rats, as one sample got lost), and 12 rats/strain of experiment 2 were tested for allele size and within-strain polymorphism by use of four markers: D2Rat118, D4Rat24, D9Rat135, and D11Rat18.

Two rats per strain (one rat from each experiment) were analyzed for product size of 16 microsatellite markers (D1Rat55, D3Rat80, D5Rat33, D6Rat160, D7Rat103, D8Rat164, D10Rat51, D12Rat76, D13Rat111, D14Rat22, D15Rat96, D16Rat67, D17Rat51, D18Rat61, D19Rat12, and D20Rat48). Product sizes of these, and many other SSLP markers, as established by the group of Howard Jacob at the Massachusetts Institute of Technology (MIT) in about 40 rat inbred strains, can be found on the internet (13). Allele sizes for BDIX, F344, and WAG substrains also are given in these lists. These total 20 markers, located on different chromosomes, were chosen because of their high degree of polymorphism. As substrains may differ in product size, these data should be considered with some caution. Also, the product size (bp) of a marker is somewhat difficult to establish exactly; it may differ a few base pairs depending on the molecular weight reference used, and the method used for separation. For this screening, agarose gel electrophoresis was used. In these gels, only molecular weight differences of more than 4% can be distinguished.

The DNA was extracted from spleen samples, using the tissue protocol of a genomic DNA isolation kit (Puregene, Gentra Systems, Inc., Minn.). As references, DNA samples from the inbred strains BDIX/Han, BDIX/Orl, F344/Han, F344/NHsd, and WAG/Rij were used (14). Twenty microsatellite markers (Rat-MapPairs, Research Genetics, Huntsville, Ala.) were used for strain identification. The polymerase chain reaction (PCR) conditions were set according to the manufacturer's protocol, with an annealing temperature of 57°C, and 1.5 m*M* Mg^{2+}.

Length polymorphisms were established after standard agarose gel electrophoresis (3% Pronarose D-I LE (Hispanagar, Burgos, Spain) in 1 × TBE (0.9 M Tris-Borate, 1 mM EDTA) buffer, and visualization by addition of ethidium bromide. As references for length, a 50-bp ladder was used (mol. wt. marker XIII, Boehringer Mannheim GmbH, Mannheim, Germany).

Experimental design. The rats were allowed a one-week acclimatization period after arrival at the Biomedical Laboratory. Thereafter, they also received a one-week acclimatization period after transfer to the isolator, before AOM injections were started.

For experiment 1, eight females of each rat strain were given subcutaneous injections of AOM; 2 animals of each rat strain did not receive any injections, and represented the control group.

For experiment 2, four female and four male rats of each rat strain were given subcutaneous injections of AOM; 2 control animals of either sex of each strain received sterile 0.9% NaCl subcutaneous injections in similar volumes and frequencies and at the same times as did test animals with similar body weight, representing the control group (sham controls).

Carcinogen administration. Azoxymethane (AOM) was purchased (Sigma Chemical Co., St. Louis, Mo.) and was diluted with sterile 0.9% NaCl to a concentration of 5 mg/ml at the Central Pharmacy of the Odense University Hospital, Odense, Denmark. The AOM solution was prepared freshly each time and stored for about one hour at room temperature before being used. Animals were given four AOM injections in total (one injection of 15 mg/kg per week) for two periods of two weeks separated by a one-week break. This break was introduced after the second AOM injection in the first experiment, on the basis of clinical signs of disease (loss of body weight, hunched back, rough coat, apathy). The injection volume was 0.4 ml at the start and 1.0 ml at the end of the experiment. The rats stayed in

the isolator until two weeks after the final AOM injection. Thus, the total residence period in the isolator was eight weeks. Thereafter, rats were housed outside the isolator until the end of the experiment.

Humane endpoints. Animals were monitored closely to detect the presence of colon tumors in due time; a clinical impression of animal health and well being was obtained daily when examining the animals for the aforementioned clinical signs of disease. Body weight was registered twice a week from the start of the first AOM injection, a visual observation score for possible fecal bleeding was given (black feces or presence of fresh red blood) and/or abdominal distention was registered (abdominal palpation to evaluate presence of abdominal abnormalities was done twice per week).

Procedures for gross examination. Animals were killed when signs of disease were observed. When one animal from one strain needed to be killed, always one of each of the other 2 strains was killed as well for reasons of comparison of latency time. As rats from the F344 and WAG strains were chosen to be killed simultaneously with the BDIX rats that harbored colon tumors, not all rats of the other strains had developed colon tumors at the time of death. The selection of the individuals from the other two strains was based, as far as possible, on positive signs indicating presence of colon tumors. In case such signs were negative for all individuals of a certain strain at a certain time point, numerical selection was performed (i.e., the rat journal number). Control animals were killed at the same time as that of the first animal with carcinogen-induced tumor and at the same time as the final animal with carcinogen-induced tumor. Animals were killed by inhalation of CO_2 after they had been anesthetized (duration maximum, 30 min) with a mixture of 0.3 ml of Hypnorm/kg given s.c. (0.095 mg of fentanyl citrate and 3 mg of fluanisone/kg; Janssen Animal Health, Beerse, Belgium) and 0.675 ml of Dormicum/kg (3.375 mg of midazolam/kg; Dumex-Alpharma, Oslo, Norway).

Immediately after death, the lungs, liver, mesenteric lymph nodes, stomach, small intestine, cecum, colon, rectum, kidneys, and spleen were examined macroscopically. Standard tissue specimens were taken from the caudal lung lobe, the left lateral liver lobe, two to three mesenteric lymph nodes, small intestine (the first three centimeters distal to the stomach), and colon (ascending, transverse, descending). Location, size and macroscopic appearance (flat versus polypoid) were registered for each tumor. Macroscopic tumors were divided into two equal parts: one for histologic examination and one for immunohistochemical analysis. Spleen specimens were sent away for genetic studies (Maria den Bieman, Utrecht University, The Netherlands).

Histologic examination. Tissue specimens were fixed in 4% (vol/vol) formaldehyde buffered with 0.075*M* sodium phosphate (pH 7) (> 48 h) and embedded in paraffin. The tissue was cut into 5-µm-thick sections and was stained with hematoxylin and eosin. Sections were cut until characterization of the neoplasms could be performed. Adenomas were classified as tubular, tubulovillous, and villous, and adenomas as well as carcinomas were graded according to the WHO classification and were staged following Dukes' classification (15, 16).

Immunohistochemical analysis. Tissue specimens were embedded in Tissue-Tek (Sakura Finetek Europa B.V., The Netherlands) and snap frozen in 1,2-methylbutane (Sigma-Aldrich, catalog No. 27,034-2, Milwaukee, Wis.) for 30 sec. Specimens were stored at -80°C until use. Tissues were cut into 4-µm-thick cryosections and air-dried overnight. Sections were fixed in acetone for 10 min, then were air dried for at least 30 min. Subsequently, the sections were washed twice with phosphate-buffered saline (PBS) and incubated for 30 min with anti-human cytokeratin 20 antibodies (clone Ks 20.8, DAKO A/S, N1627) undiluted or anti-rat CD45 antibodies (clone MRX OX-1, BioSource, ARS4501) diluted 1:800 with PBS containing 1% bovine serum albumin (BSA). After rinsing three times with PBS, the sections were further incubated for 30 min with 1:20 PBS-diluted rabbit anti-mouse peroxidase-conjugated antibodies (DAKO, catalog No. P260) in 1% BSA and 50% normal rat serum (Harlan, Horst, The Netherlands) to diminish non-specific staining. A third 30-min incubation was performed with 1:20 diluted swine anti rabbit peroxidase-conjugated antibodies (DAKO, cat. No. P217) to intensify staining. Sections were developed by incubation for 10 min with a DAB tablet (DAKO, Chromogen S3000) dissolved in PBS containing 0.0064% H_2O_2, which was filtered once before use. Sections were slightly counterstained with hematoxylin and mounted in glycerin gelatin.

Statistic calculations. Data on tumor frequency were fitted into a logistic regression model to judge the effects of strain, sex, and experiment (Intercooled Stata 6.0; Stata Corporation, College Station, Tex.). A value of $P < 0.05$ was considered significant.

Table 1. Body weight (mean ± SD) of the rats of the two experiments

Experiment 1			Body weight (g)	
Strain	Sex	Time	AOM treated	Control
			(n = 8)	(n = 2)
BDIX	F	Start	206 ± 9.0	207 ± 15.6
		End	232 ± 8.9	241 ± 11.3
F344	F	Start	161 ± 6.7	152 ± 5.7
		End	224 ± 8.8	224 ± 9.2
WAG	F	Start	146 ± 6.9	148 ± 2.8
		End	198 ± 7.8	211 ± 6.4
Experiment 2			(n = 4)	(n = 2)
BDIX	F	Start	180 ± 4.7	173 ± 10.6
		End	210 ± 6.1	231 ± 10.6
	M	Start	237 ± 9.3	233 ± 37.5
		End	361 ± 17.6	358 ± 33.2
F344	F	Start	140 ± 13.6	135 ± 0
		End	222 ± 17.8	232 ± 6.4
	M	Start	197 ± 10.3	200 ± 8.5
		End	386 ± 16.7	417 ± 24.7
WAG	F	Start	126 ± 7.8	126 ± 5.7
		End	195 ± 12.5	200 ± 8.5
	M	Start	203 ± 9.8	178 ± 7.1
		End	327 ± 20.0	326 ± 25.5

Results

Humane endpoints. Individual weight loss > 5% was not observed in any rat. Body weight of the AOM-treated rats was virtually similar to that of the control rats throughout the experiments (Table 1). This accounted for all three inbred rat strains in both experiments. Reliable statistical evaluation between the AOM-treated and control rats within the same strain could not be performed, as only two control rats were used. Temporary decrease in body weight occurred in the BDIX strain in experiment 1 after the second AOM injection, which was one of the reasons that a one-week break in the AOM treatments was introduced. In the second experiment, this effect of AOM was not apparent. However, the one-week break was maintained for reasons of comparison. Even in the final part of the experiment

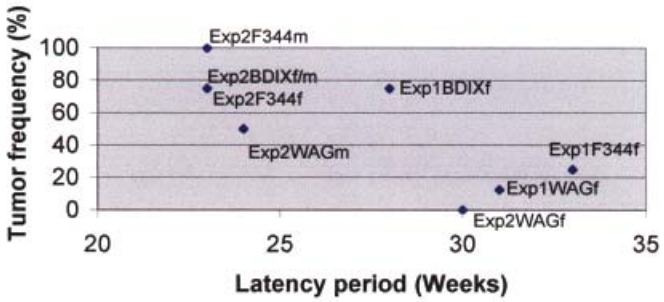

Figure 1. Tumor frequency and latency period (f = females; m = males). In the case of tumor frequency of 0%, the latency period indicates the final week where no tumors were observed.

when rectal bleeding was registered and big tumors were found on palpation and at necropsy, decrease in body weight was not evident. Therefore, the decision to kill an animal was mainly made on the basis of signs of rectal bleeding and/or results of palpation of the abdomen, along with the overall physical appearance.

Colon tumor frequency and minimum latency period. Colon tumor frequency (number of rats with tumor[s]/total number of rats in the entire experimental group × 100%) versus shortest latency period (time from the first AOM injection to the first detection of colon tumors [in weeks]) is presented in Fig. 1. All tumors identified macroscopically were subsequently identified by microscopic examination. Only colon adenomas and adenocarcinomas were included in the final evaluation.

In experiment 1, colon neoplasms developed in six of eight (75%) BDIX female rats, two out eight (25%) F344 female rats, and one of eight (12.5%) WAG female rats. In experiment 2, colon neoplasms developed in three of four female (75%) and three of four male BDIX rats (75%), two of four (50%) female and all male F344 rats (100%), and none of four female (0%), but two out of four male WAG rats (50%).

Statistical evaluation revealed a significant difference among the three strains ($P = 0.01$). The F344 strain had lower colon tumor frequency than did the BDIX strain, which was non-significant ($P = 0.24$). The WAG strain had a significantly lower frequency than did the BDIX ($P = 0.003$) and the F344 ($P = 0.03$) strains. Male rats had a higher frequency than did females, although the difference was not statistically significant ($P = 0.15$). There was no significant difference between results of the two experiments ($P = 0.41$). Control rats of both experiments did not have macroscopically or microscopically detectable changes in the colon or other organs.

The shortest latency period was defined as the period between the first AOM injection and the earliest registered development of the neoplastic alterations in the colon. In experiment 1, the first tumor was observed in the BDIX rat strain 28 weeks after the first AOM injection (Fig. 1). The latency period for observation of adenocarcinoma in the F344 and the WAG rat strains was 33 and 31 weeks, respectively.

In experiment 2, the first colon tumor was observed 23 weeks after the first AOM injections in male and female BDIX rats. The shortest latency period for observation of colon tumors in the F344 males and females was 23 weeks. The WAG rat strain had a minimum latency period for males of 24 weeks and for females of 30 weeks.

Number of tumors per animal and colon tumor size. The number of tumors per animal differed from one to four in the BDIX rats. The F344 and WAG rats had minor number of tumors per animal, two to three and one to two, respectively. In case of multiple colon tumors, the macroscopical phenotypic appearance often differed between individual tumors (Fig. 2). Tumor diameters varied from 0.1 cm up to 1.0 cm. In both experiments, the BDIX strain had the largest diameter (7 to 8 mm). The largest diameter of the tumor in the F344 and WAG strain was about 4.5 and 3.5 mm, respectively. The diameter was measured, using a ruler under the microscope on histologic section.

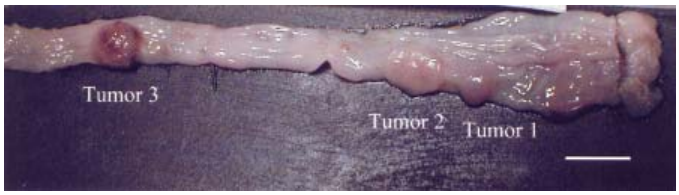

Figure 2. Macroscopic appearance of three colon tumors (bar = 1 cm).

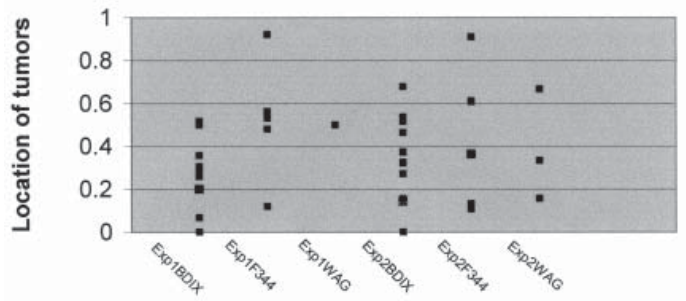

Figure 3. Location of colon and rectal tumors of the rats of the study. Location is measured in percentage (%) of total colon length, starting from the anus; thus, the value for the anus is 0% and for the cecum is 100%.

Other tumors. Small intestinal neoplasms were observed in two of eight of the WAG rats in experiment 1 and in three of eight F344 rats (one female and two males) in experiment. 2 at a spot two to three centimeters distal to the stomach. A renal neoplasm was observed in one case (male WAG rat in experiment 2) and a hepatic neoplasm in another (male WAG rat in experiment 2).

Histopathologic observations. The location of tumors found in the colon and rectum is illustrated in Figure 3. Most of the tumors were located in the distal part (descending, sigmoid, and rectal colon). Polypoid and non-polypoid (flat) lesions were observed in this model. Polypoid lesions were defined as elevated lesions surrounded by apparently normal colon mucosa. Non-polypoid lesions were defined as well-circumscribed mucosal changes that did not protrude into the lumen of the gut. Adenomas and adenocarcinomas (Fig. 4) were observed in this model.

In experiment 1, all adenomas were tubular. The dysplasia varied from moderate (40%) to severe (60%). All carcinomas were moderately differentiated, except one tumor, which was classified as highly differentiated. In experiment 2, all adenomas were tubular, except one, which was tubulovillous. The dysplasia varied from mild (25%) to moderate (50%) to severe (25%). Most carcinomas were moderately differentiated (70%), the remaining (30%) being highly differentiated. Remnants of adenoma, defined as dysplastic epithelium over intact lamina propria and muscularis mucosae, were present in only one of the carcinomas.

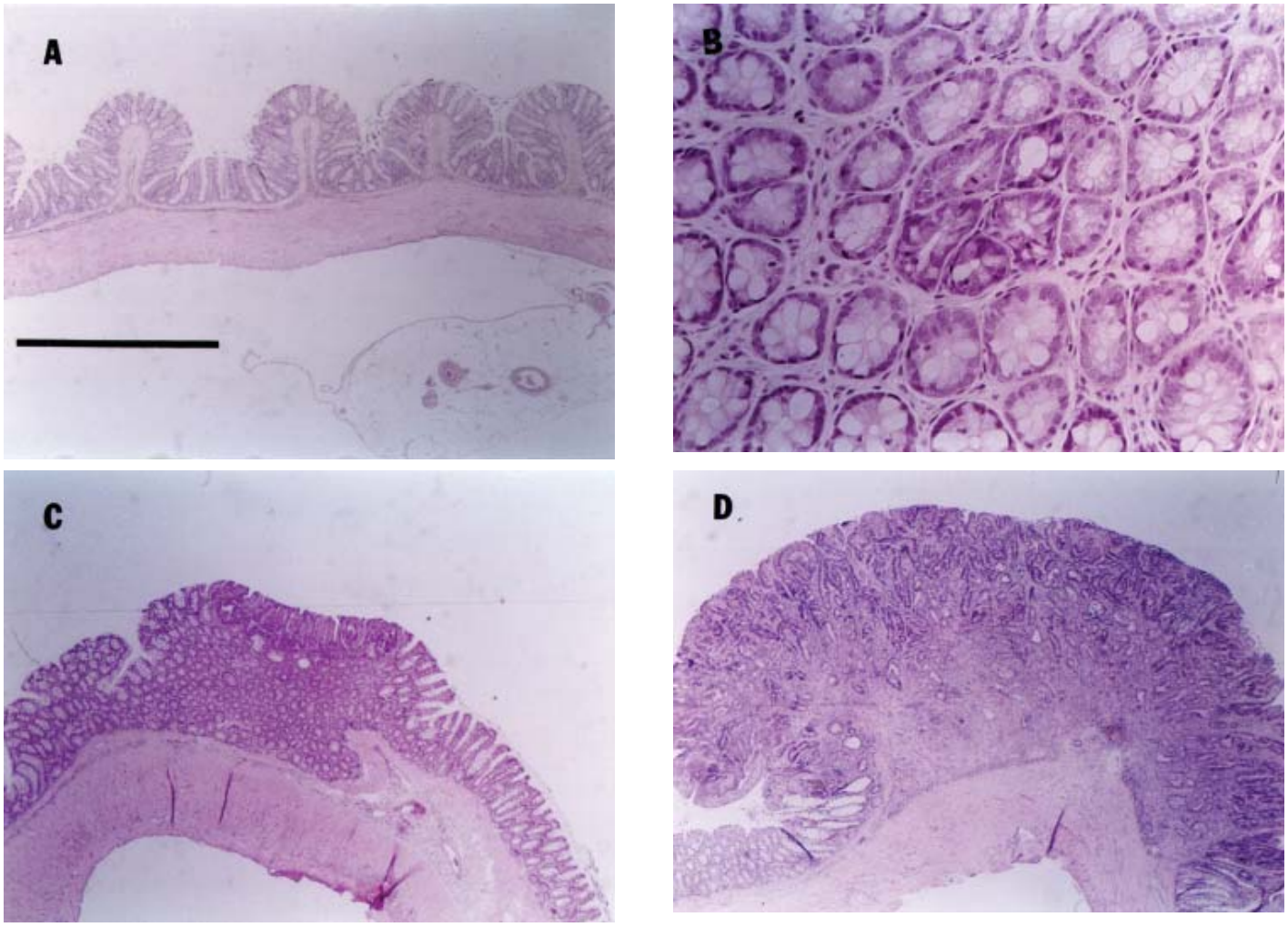

Figure 4. Various stages in the development of colon tumors. A = normal colon epithelium; B = aberrant crypt foci; C = adenoma stage; and D = carcinoma stage. Hematoxylin and eosin stain; bar = 1 mm (A, B, and D) magnification = 117× (B).

Nearly all carcinomas had an irregular invasive front, and only one had a pushing margin. The invasion was always in the submucosa, and only one carcinoma had tumor cells in the tunica muscularis, all thus being Dukes' type A. Perineural or vascular invasion was not present. In all instances, invasion was followed by a pronounced desmoplastic reaction and an inflammatory infiltrate dominated by neutrophilic and eosinophilic granulocytes, and in some instances, followed by a less pronounced infiltration with lymphocytes and plasma cells. No tumors contained intra-epithelial lymphocytes or granulomas (Fig. 4).

Immunohistochemical analyses. All intestinal tumors in the rats of all three inbred strains were positive for cytokeratin 20 (Fig. 5). In non-neoplastic as well as neoplastic mucosa, $CD45^+$ cells were present in the lamina propria. In malignant neoplasms, there was a wide inter- and intra-tumor variation in the number of leukocytes present. This presence of leucocytes was mainly confined to the stroma of adenocarcinomas. Difference was not observed among the three inbred strains (Fig. 6).

Genetic analysis of the strains. For identification of the strains, we compared the polymerase chain reaction (PCR) product sizes of two samples per strain with the product sizes of the reference strains (results not shown). Product sizes were compared with the data given in the Research Genetics database (17). Neither within-strain, nor between-experiment polymorphism was detected. The phenotypes of the three inbred strains were in line with previously established phenotypes for these strains. The product size for each of the three inbred strains was identical to that of the appropriate reference strain for each of the 20 markers. Within the limits of agarose gel electrophoresis, the estimated sizes were identical to product sizes of the same strains as given in the Research Genetics Database.

Discussion

To have control over the genetic background within each strain, as well as controlled genetic variation, three inbred rat strains were chosen (WAG, BDIX, and F344) (18). The three inbred rat strains were selected on basis of the availability of established syngeneic colon cancer cell lines. In our experiments, there was a significant variation in colon tumor frequency among the inbred rat strains, which presumably was due to a genetic variation in susceptibility to the carcinogen. This variation could be due to several parameters differentially affecting conversion of the procarcinogen (AOM) to the final carcinogen, (e.g., differences in enzyme activity in the liver).

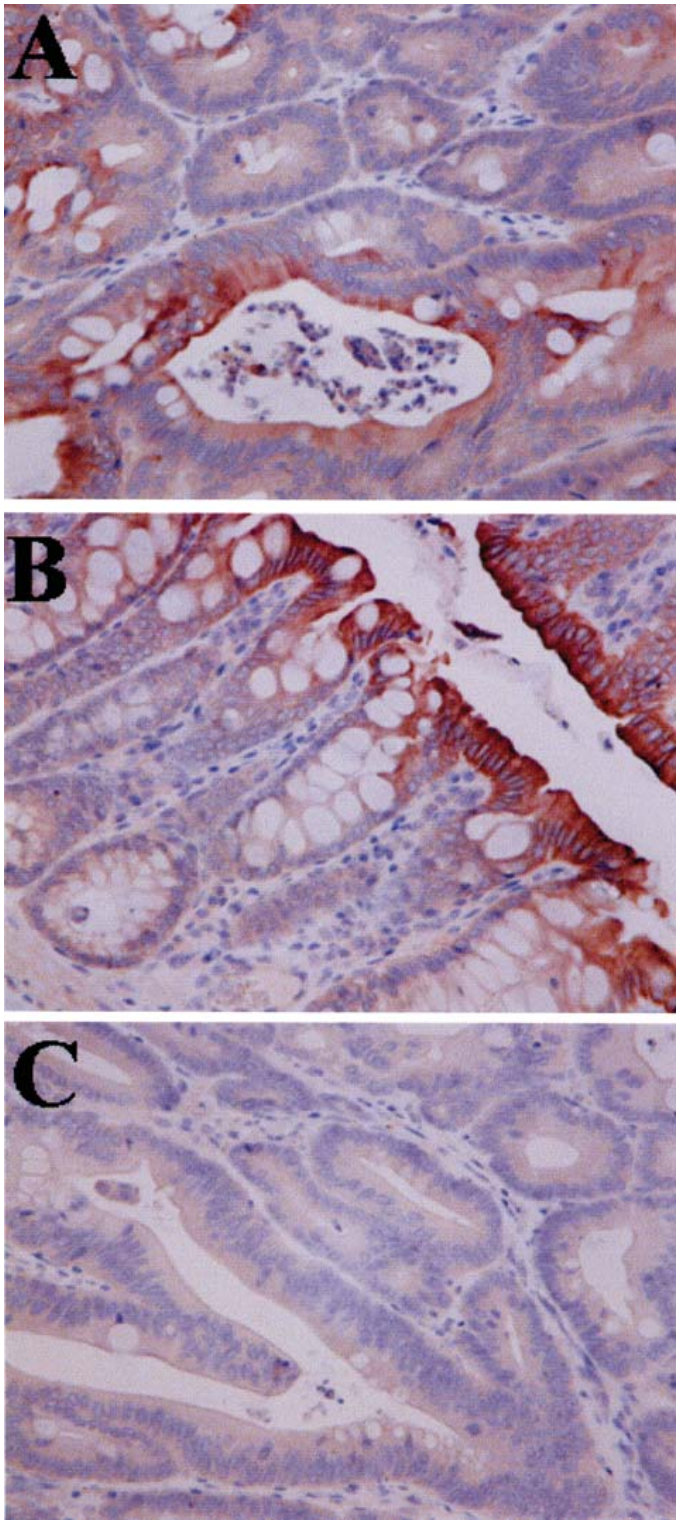

Figure 5. Cytokeratin 20 staining of colonic tumor tissue (A), normal colon epithelium (B), and negative isotypic control tissue (C). Magnification 176×.

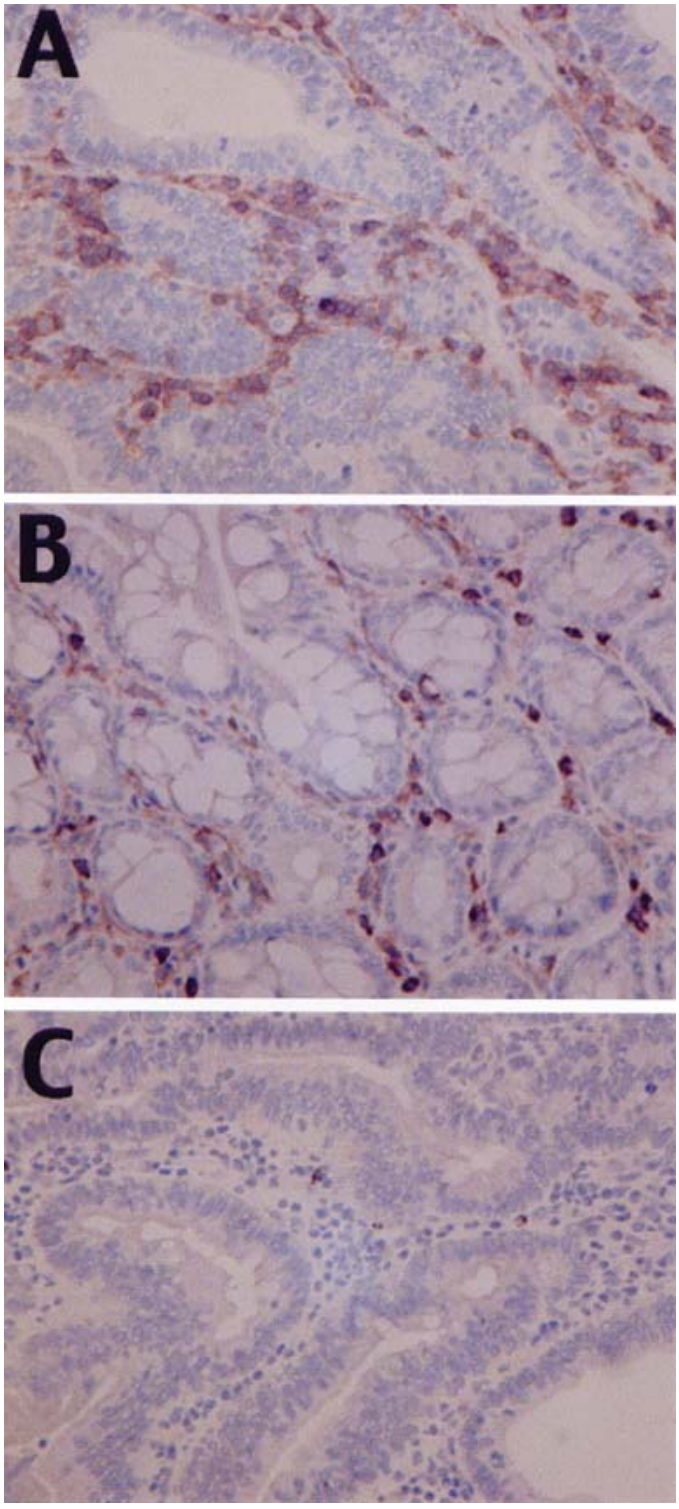

Figure 6. Infiltration of $CD45^+$ cells in colonic tumor tissue (A), normal colon epithelium (B), and isotypic control (C). Magnification 218×.

In both experiments, individuals from and within each inbred strain were genetically similar. This was verified by molecular biological characterization of a spleen DNA sample from each animal. The phenotypes of the three inbred strains were in line with earlier established phenotypes for these strains. Therefore, we can conclude that there was no detectable genetic variation within the strains or between the two experiments. The variation in the phenotype of the colon tumors within individuals of the same strain must, therefore, be related to non-genetic factors.

When comparing body weight change in the AOM-treated rats with that in controls in all three inbred rat strains, a clear

effect of the AOM-injections on body weight in either of the two experiments was not seen. Although there was a temporary decrease in body weight after the second carcinogen treatment in the first experiment, this trend did not continue. This was also the result of the decision to introduce a one-week break in between the AOM treatments, to ensure survival of all animals. Although rectal bleeding developed and large colon tumors were observed during the final stage of the study, decrease in body weight of the rats was not apparent. Therefore, rectal bleeding and findings of abdominal palpation rather than body weight loss should be considered as more reliable markers for the presence of colorectal cancers in these rats. Even though large colon tumors appeared (up to diameter of 1 cm), ileus did not develop and no rats died "spontaneously" (i.e., before the decision was taken to perform euthanasia). As body weight loss was not a good indicator, use of (severe) body weight loss as a guideline for humane endpoints for experimentally induced colorectal cancer models is questionable. New methods for defining humane endpoints are requested (e.g., endoscopic inspection of the colon and rectum or molecular analyses of fecal samples for various oncogenic markers).

This study indicated that four AOM injections of 15 mg/kg are sufficient to induce colorectal cancer in reproducible manner, at least in the BDIX rat strain. The BDIX strain had a reproducible high tumor frequency in both experiments and either sex and the shortest latency period. It is not clear why the F344 rat females had large differences in tumor frequency and minimal latency period between the two experiments; this may be the result of the experimental design and/or the result of unknown interfering factors. A longer latency period in F344 and Wag rat strains could be reasons for the low tumor frequency found in our study, as determined by the experimental design. To make a fully standardized comparison of the three inbred strains, one would have to euthanize animals of the three strains simultaneously at one or more determined times; however, this was outside the scope of the study because the goal was to identify a strain that develops large tumors in a short latency period in reproducible manner.

Only macroscopically visible tumors were dissected out and analyzed in this study. Furthermore, few histologic sections were evaluated for the presence of only microscopically detectable changes, and therefore, minor neoplastic alterations could have been missed. Neoplastic alterations were not found in macroscopically "normal" tissue. However, as it was our primary goal to find tumors of about one centimeter, attempts were not made to detect all minor neoplasms microscopically.

In this study, when tumor diameter up to one centimeter is considered necessary for future liver metastases model studies, only the BDIX rat strain was suitable for our purpose under these experimental conditions.

Despite the low number of animals used, a sex difference in their susceptibility to the carcinogen was observed in the WAG and F344 rat strains, but not in the BDIX rat strain. As no sex difference is seen in the human situation, this probably makes the BDIX rat strain a more relevant model. The sex differences may indicate that sex hormones interact with metabolism of the procarcinogens in the WAG and F344 strains.

Histopathologic characterization of the colon tumors in all three rat strains indicated a close resemblance to human histopathologic changes. The colorectal tumors in this model are similar to those in humans, located mainly in the distal parts of the colon (descending and sigmoid colon and rectum) (Fig. 4). Polypoid and non-polypoid neoplastic lesions were observed and were characterized as adenomas and carcinomas. Most of the carcinomas were moderately differentiated, which is in accordance with the human situation. As illustrated (Fig. 4), normal colonic epithelium (Fig. 4A) is similar to that of humans. In accordance with the multi-step colon neoplasia hypothesis (19), this rat model has all phenotypic steps in the multi-step process: aberrant crypt foci (Fig. 4B), the adenoma stage (Fig. 4C), and adenocarcinomas (Fig. 4D).

The carcinomas only invaded the submucosa; thus all corresponded to the human Dukes' A type. This could also explain the low frequency of metastases in our rat model, as this is a rare observation in patients with Dukes' A type tumors.

Invasion was followed by an inflammatory response mainly due to infiltration with neutrophilic and eosinophilic granulocytes, and to a lesser degree by infiltration of lymphocytes and plasma cells. This inflammatory response reflects the situation with human colorectal cancers. The histologic appearance of the tumors corresponded well to the human counterpart, bearing closest resemblance to the microsatellite stable variant, which accounts for about 85% of the tumors in these patients (16).

All tumors were of epithelial origin and were positive for cytokeratin 20 ($CK20^+$), which is typical for colon epithelium. Although we used the human marker for $CK20^+$, the results were considered reliable, as the cytokeratins are conserved throughout evolution and species. We assumed that rat CK20 antibodies, which are not commercially available, resemble human CK20 antibodies.

As this model is planned to be included in future immunotherapy studies, the immunologic status of the tumors was analyzed. There was a presence of $CD45^+$ cells in the tumor stroma, compared with that of normal colon lamina propria and submucosa. This may indicate increased immunologic responsiveness toward the tumor cells. Therefore, AOM-induced colon cancers in these three inbred rat strains are considered suitable to use when studying immune therapy, as the presence of lymphocytes and plasma cells indicates that the immune system can be activated.

In conclusion, under our experimental conditions, large differences existed between different rat strains when inducing colon tumors with AOM. The AOM injections in BDIX rats lead to a reproducible high frequency of colon tumors of large size after a short latency period. Histologic aspects also closely resembled those of the human process. It can be concluded that weight loss in carcinogen-treated rats was not a reliable humane endpoint in our experimental design. We have introduced other parameters (e.g., rectal bleeding and results of palpation of the abdomen), but other parameters such as endoscopic inspection of the colon are necessary to obtain a more accurate assessment of the presence of tumors and their humane endpoints.

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