Carcinogenesis vol.10 no.10 pp.1847–1854, 1989

ICRC mouse with congenital mega-esophagus as a model to study esophageal tumorigenesis

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ICRC mouse, an inbred strain, developed at the Cancer Research Institute, Bombay, exhibits mega-esophagus with markedly hyperplastic mucosa. Diethylnitrosamine (DEN) when given in drinking water at the dose of 4 mg/kg body weight/day, induced esophageal papillomas consistently in 100% of the animals, in a short period of 12 weeks. Further, tumors were produced, even at a very low cumulative dose of 28 mg/kg body weight. Development of the esophageal papillomas was dose dependent. DEN even induces esophageal tumors transplacentally in the ICRC F_1 progeny. Tobacco acts predominantly as a promoter in this system. ICRC mouse thus provides a much needed animal model to study esophageal tumorigenesis, including the two-stage carcinogenesis. An interesting feature of the study is that initiation could be induced by exposure to low doses of DEN in the intra-uterine life. Tumors develop in such F_1 animals only if they are fed tobacco, a predominant promoter, post-natally.

Introduction

Esophageal cancer is a common cancer in many parts of the world, including India. Its etiology and pathogenesis are still poorly understood. Epidemiological studies suggest that environmental factors, including consumption of alcohol and tobacco, and nitrosamines, play an important role in the etiology of the cancer (1). Amongst the host factors, achalasia, in which constriction occurs at the gastro-esophageal junction, causing retention of food in the esophagus and consequent chronic irritation of the esophageal mucosa, is considered, by several workers, to be a predisposing factor that can lead to esophageal carcinoma in humans (2,3).

Cell proliferation plays a key role in induction of tumors (4). Increased cellular proliferation, as seen in regenerating tissues, could promote tumor development. Thus, it has been shown that, as compared to the normal, the regenerating liver is more prone to chemical carcinogenesis (5). Mucosal hyperplasia, observed in achalasia, may be responsible for the higher incidence of cancer in this condition.

Since 1957, an inbred strain of large-sized albino mice, named ICRC/HiCri (ICRC), has been maintained by the Cancer Research Institute, Bombay (6,7). The animals were originally obtained from Haffkine Institute, Bombay, as a random-bred colony. Inbreeding resulted in the development of a strain that showed spontaneous mammary cancer and leukemia. In addition, all adult animals of both sexes also show mega-esophagus in which mucosa is markedly hyperplastic (7). The condition appears at ~3 weeks, but becomes evident at 3 months of age. In view of the promotional effects of regeneration in chemical carcinogenesis, it was felt that the ICRC mouse may readily develop esophageal tumors on challenge with a suitable carcinogen.

Chemical carcinogenesis is considered to be at least a two-step process, consisting of the stages of initiation and promotion. Initiation could be induced even by exposure in the intra-uterine period (8). F_1 and F_2 progeny of mothers exposed to carcinogens develop tumors (9). Further, tumor incidence is increased if such offsprings are also exposed to promoters post-natally. In this study, we have shown that the ICRC mouse is a good model to study the two-stage, as well as transplacental, esophageal carcinogenesis. In this model, tobacco which is a known risk factor for both oral and esophageal cancer (10), has been found predominantly to be a promoter.

Materials and methods

Animals and treatment

Adult inbred ICRC mice were used in the study. Each animal was housed separately in a cage and fed standard laboratory diet and water *ad libitum*. The starting weights of the adult animals were 30–33 g. Diethylnitrosamine (DEN*) was administered in different doses, depending on the experimental design, in drinking water, noting daily consumption and thereby adjusting the dosage.

Four sets of experiments were conducted to test: (i) dose–response effects of DEN; (ii) modifying influence of age on DEN-induced tumors; (iii) transplacental effects of DEN on the esophagus of the ICRC offspring; and (iv) modifying effects of tobacco on DEN-induced tumorigenesis. In all the post-natal carcinogenesis, DEN was fed at the level of 4 mg/kg body weight/day. In the first set of experiments, 2-month-old ICRC mice were fed DEN daily, and groups of animals killed at 1, 2, 3, 4½ and 6 months. It was observed that all animals developed esophageal tumors beyond 3 months. Subsequently, to determine its minimum effective cumulative dose, DEN was fed for only 1, 2, 4 and 8 weeks, giving cumulative doses of 28, 56, 112 and 224 mg/kg body weight respectively. The animals were killed at 3 and 6 months.

To study the modifying influence of age, a group of 15, 21-day-old weanling mice (11–14 g), were given DEN (4 mg/kg/day), in drinking water and killed at 4½ months. The results were compared with animals treated similarly to those in Experiment 1.

The third series of experiments had two sub-groups. In one of them, DEN was administered to the pregnant mice for a continuous period of 7 days, immediately on pregnancy, in its second or third week. Based on preliminary studies 40 mg/day/kg body weight (cumulative dose 280 mg/kg body weight) of DEN was fed to pregnant females in drinking water. The control group of pregnant animals did not receive DEN. Offspring were reared in normal conditions and no carcinogen was fed to them in the post-natal life. They were killed at 8–9 months of age. A number of animals developed tumors. The best results were obtained in the offspring of mothers fed DEN in the second week. In the other sub-group, therefore, mothers received 20 mg DEN/kg/day in the second week of pregnancy. When the offspring reached 10 weeks of age they were given either normal diet or a diet containing 5% tobacco, and killed 6 months later. This dose of DEN was chosen on the basis of preliminary studies which had shown that, at this level, no tumors were produced in the F_1 animals.

In the fourth set of experiments, dry, powdered tobacco (*Nicotiana tobaccum*) was mixed in the normal diet at a concentration of 5%. A locally popular brand known as Pandharpuri chewing tobacco was used. The mixture containing tobacco plus food was compressed in pelleted form and fed to the 2-month-old animals. They were divided into three groups. Animals in Groups I and II were fed DEN (4 mg/kg/day) for 2 months and 2 weeks respectively. Thereafter, for the next 3 months, half the animals from each group were fed normal diet, and the other half tobacco-containing diet. The third group of animals were fed normal diet for the first 2 months, followed by tobacco-containing diet for the next 3–3½ months. All animals were killed at the end of the study.

*Abbreviations: DEN, diethylnitrosamine; H&E, hematoxylin and eosin.

The χ^2 test was used for comparison of proportion of tumor-bearing animals; however, when the cell frequencies were small, Fischer's exact test was applied. For comparison of mean values, Student's *t*-test was used.

Histopathology

Animals were killed by cervical dislocation at intervals according to the experimental design. A gross examination of all organs was carried out. The esophagus was dissected completely open on a filter paper so that it remained flat, and then fixed in Bouin's fixative. Longitudinal sections were taken from the junctions of the upper and lower one third to the middle one third. In addition, sections were taken from papillomas. Paraffin sections cut at 5 μ were stained with hematoxylin and eosin (H&E).

Results

The ICRC mouse and general appearance of esophagus

The pedigree chart of the ICRC mouse is shown in Figure 1. On inbreeding, this animal showed peculiar characteristics. From the fifth generation onwards the strain exhibited spontaneous mammary tumors and leukemia. The tumors were seen in ~25–30% animals at the age of 10–12 months. In the 14th generation, in addition, ~8% animals of both sexes also showed mega-esophagus, which was seen in 100% animals after the 60th generation. In this condition, a constriction occurs at the gastro-esophageal junction due to failure of the muscles to relax after each contraction. Due to this constriction, ingested food cannot pass down into the stomach. The stagnation of food in the esophagus results in dilation of upper esophagus. The condition occurs at ~3 weeks, and becomes evident at 2–3 months of age. The condition was seen progressively in larger numbers of animals and by the 60th generations, 100% animals showed mega-esophagus. The incidence of spontaneous breast tumor and leukemia has developed to 33 and 8% respectively. At present the ICRC strain is in the 80th generation.

The gross appearance of the esophagus and stomach of the animals at different ages is shown in Figure 2. Although some dilation of the middle one third is observed even at 3 weeks, the esophagus is grossly dilated only at 4 months and later. The extent of dilation varied from animal to animal. Dilatation affects almost the whole length, except in small portions of the proximal and distal segments. Esophageal mucosa in a normal adult mouse (Swiss) is lined by stratified squamous epithelium, three or four cell layers thick, showing orderly keratinization (Figure 3a). Mitotic figures are hardly ever seen. In the ICRC mouse of comparable age, the mucosa is markedly hyperplastic, consisting of six to eight cell layers, associated with hyperkeratosis and parakeratosis (Figure 3b). Mitotic figures are frequently observed in the basal cell layer. In some animals, foci of lymphocytic infiltration are seen in the sub-mucosa. The muscular coat is markedly hypertrophied. Ulceration, associated with chronic inflammation of the sub-mucosa, is observed in some animals at 8 months of age. We have so far examined >100, 8-month-old animals, but no papilloma has been seen in any of them.

General observations

At the start of the experiment the mean weight of the animals was 31 g (Figure 4). Control animals progressively gained weight throughout the study. The DEN-fed animals maintained body weight for the first 3 months, but thereafter lost weight. However, even at the end of the study, the weight loss was ≤2–4 g.

Dose response of DEN

Esophagi of adult animals showed grossly no lesion up to 2 months of DEN feeding. However, by the third month, 100% of the animals developed tumors (Figure 5). An average of 10 papillomas per esophagus was seen. With increasing duration and higher cumulative dose of DEN, there was a progressive increase

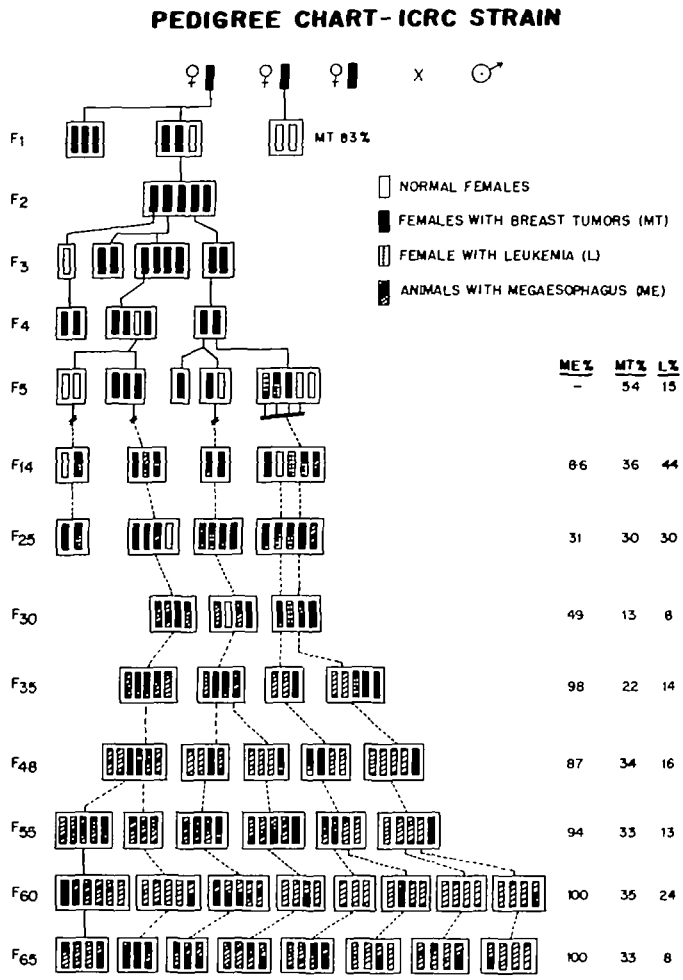

Fig. 1. Pedigree chart of ICRC mouse.

in the number of papillomas. The differences between 3 and 6 months were statistically significant. At 6 months, the number was so large that it interfered with nutrition and the animals frequently became moribund. Hence, the experiments were terminated at this stage. None of the other organs, which included stomach, liver, small intestines, lung, spleen and kidney, showed any other abnormality or tumors. No comments could be made on the effects of DEN on the incidence of the spontaneous tumors, generally seen only in the animals >10 months old, as even in the maximal-duration experiments the animals were killed at 8 months. Further, at a cumulative dose as low as 28 mg/kg body weight, papillomas were induced in 6 months (Table I). The differences in DEN-dosage effect were specially observed at 3 months, with 28- and 56-mg cumulative-dose animals not showing any tumors, and 112- and 224-mg cumulative-dose animals, all showing two to six tumors. At the end of 6 months, a progressive increase in the number of papillomas was seen as the cumulative dose increased from 28 to 112 mg but thereafter it plateaued. The mean number of papillomas in animals treated with DEN up to 2 weeks was significantly lower ($P < 0.001$) than those treated for 4 and 8 weeks.

Age-dependent modulations

The average number of papillomas per esophagus in 21-day-old animals continuously fed DEN for 4½ months was 31.9 ± 2.09. This was 1.8 times the number seen in similarly treated adult animals (Figure 5). The differences were statistically significant ($P < 0.001$).

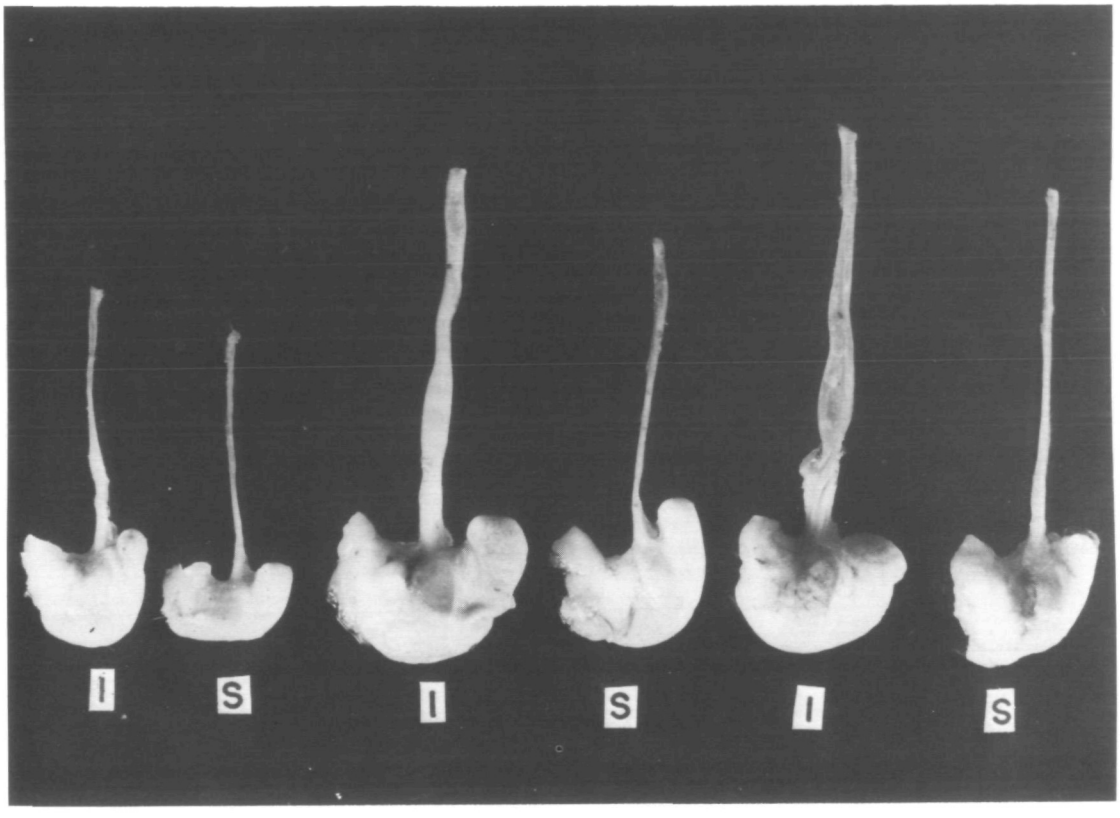

Fig. 2. Gross appearance of esophagus and stomach of ICRC (I) mice at different ages of 21 days (LHS), 4 months (middle) and 8 months (RHS), compared with Swiss (S) mice of similar age.

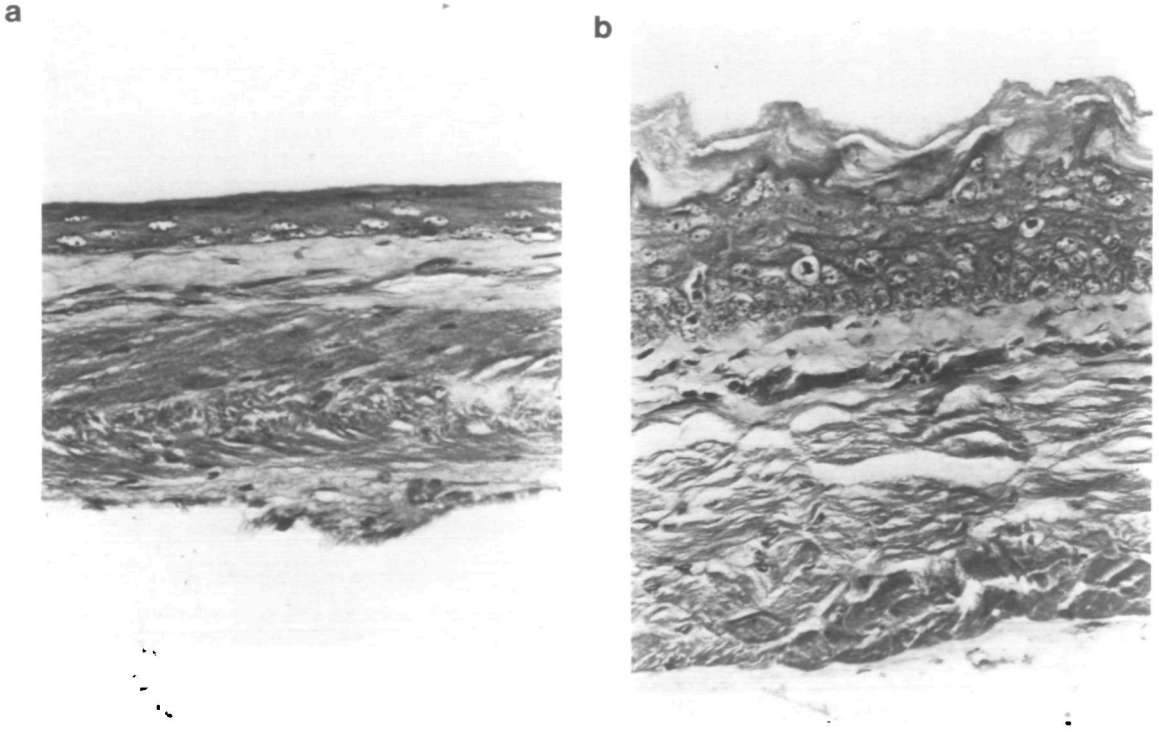

Fig. 3. (a) Photomicrograph of a section of the esophagus of a normal adult Swiss mouse showing the mucosa which is two or three cells thick. No mitotic figures are seen (H&E; ×300). (b) A section of the esophagus of a normal adult ICRC mouse. The mucosa is hyperplastic and seven or eight cell layers thick. The basal cell layer shows a few mitoses. There is also hypertrophy of the muscular coat (H&E; ×300).

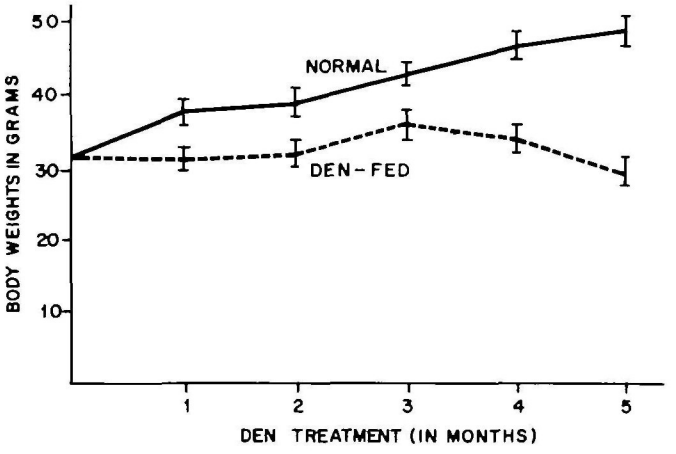

Fig. 4. Weight chart.

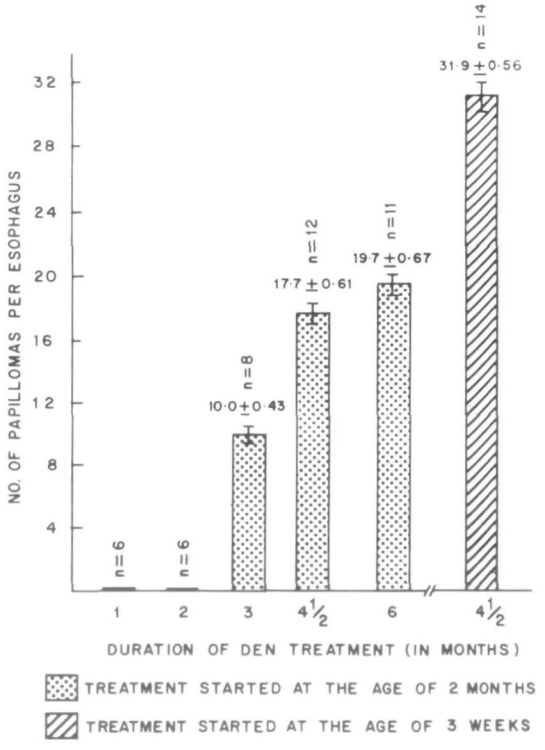

Fig. 5. Histogram showing the number of esophageal papillomas in animals fed DEN for different durations.

Transplacental effects of DEN

The offspring in the control group did not develop esophageal tumors, whereas one or two papillomas were observed in offspring of DEN-fed mothers (Table II). The tumors were observed in 30, 66.6 and 42% of the offspring whose mothers had received DEN in the first, second or third week of pregnancy respectively. The DEN-fed mothers, in all the three groups, showed one or two papillomas per animal at 8 months of age, when they were killed. The differences were found statistically significant when F_1 progeny of the DEN-fed animals was compared as a group with the control animals. However, the differences within the various groups of DEN-fed progeny were not significant.

When DEN was fed at low dose (20 mg/kg/day), in the second week of pregnancy, and progeny later fed normal diet, no tumors were seen. However, if the offspring were given a diet containing tobacco, tumors developed in 55% of animals. The differences were statistically significant ($P = 0.01$; Table III).

Effects of tobacco

The animals fed only DEN for 2 months showed three to six papillomas per esophagus (Table IV). However, when DEN treatment was followed by feeding of 5% tobacco diet, the average number of papillomas increased 5-fold, ranging from 18 to 25 papillomas per esophagus. The difference in the number of papillomas was highly significant ($P < 0.001$). With DEN treatment of only 2 weeks, followed by normal diet, no tumors were observed in the animals. However, after a 2-week DEN treatment followed by a diet containing 5% tobacco, all the animals (10/10) showed the presence of two to six tumors. The group of animals fed only 5% tobacco with no prior treatment of DEN, did not show any papillomas during the same period.

Histopathology

Papillomas appeared on the entire surface of the esophagus. Their density showed a gradient from proximal to distal end, with the maximum number of papillomas in the lower-one-third region. To begin with, they appeared to be small elevations of the mucosa. But with time, they grew in size and showed a definite pedicle and became pedunculated. The gross appearance of papillomas in animals fed DEN for 4½ months is shown in Figure 6. At this stage a very large number of papillomas, many of them with ulceration, were seen.

Although the timing of the appearance of papillomas and their number differed in different experiments, the morphology of the papillomas was similar. Morphologic changes are therefore described together. The papillomas in the esophagus showed squamous-cell differentiation. The histopathological changes

Table I. Dose–response effect of DEN

Duration of DEN[a] treatment (in weeks)	Cumulative dose (mg/kg body wt)	Animals killed after					
		3 months			6 months		
		No. of animals	No. of papilloma-bearing animals	Mean no. of papillomas/ tumor-bearing animal ± SE	No. of animals	No. of papilloma-bearing animal	Mean[b] no. of papillomas/ tumor-bearing animal ± SE
1	28	8	0	–	12	6	2.2 ± 0.61
2	56	10	0	–	8	8	3.4 ± 0.42
4	112	12	12	2.9 ± 0.35	8	8	11.4 ± 0.67
8	224	8	8	4.6 ± 0.39	9	9	10.3 ± 0.43

[a]DEN was fed at 4 mg/kg/day.
[b]$P < 0.001$ (comparing the combined groups of 1-week and 2-week treatment, with the combined group of 4-week and 8-week treatment).

involved, exclusively, areas of the surface epithelium, and were characterized by hyperkeratosis, keratin pearl formation, basal cell hyperplasia, increased thickness of the epithelium, and papillomas which showed high cellular proliferation, evident by the frequent presence of mitotic figures (Figure 7a and b). Foci of lymphocytic infiltration were frequently observed in the underlying submucosa. Ulceration, with associated chronic inflammation, was particularly seen in large tumors (Figure 7a). None of the tumors extended into the muscular coat, and no distant metastasis was observed. However, in some papillomas, cells exhibited loss of polarity and occasional groups of tumor cells invaded the underlying tissue (Figure 7c). The changes were consistent with carcinoma *in situ* or micro-invasive cancer. In a rare animal frankly invasive tumors, without any lymph node metastasis, were also seen after prolonged DEN feeding. However, the dominant growth was a well-differentiated squamous cell papilloma.

Table II. Tumor incidence of offspring of ICRC mice treated with DEN prenatally

Period of prenatal DEN[a] feeding	Total no. of offspring	No. of papilloma-[b] bearing animals	No. of papillomas/ tumor-bearing animal (range)
First week	10	3 (30.0%)*	1.0
Second week	9	6 (66.6%)**	1–2
Third week	12	5 (41.7%)***	1–2
Control (no DEN)	12	0	

[a]DEN was fed at 40 mg/kg/day for 7 days in drinking water to the pregnant mice. Offspring were killed at the age of 8 months.
[b]Significantly different from control group at: *$P = 0.16$; **$P = 0.003$; ***$P = 0.04$.

Table III. Effect of post-natal tobacco feeding on pre-natally DEN-fed ICRC offspring

Period of pre-natal DEN[a] feeding	Post-natal treatment	Total no. of offspring	No. of papilloma-bearing animals	Mean no. of papillomas/ tumor-bearing animal ± SE
Second week	Normal diet	11	0	–
Second week	5% tobacco diet	11	6[b]	1.33 ± 0.33

[a]DEN fed at 20 mg/kg/day for 7 days in drinking water to the pregnant mice.
[b]$P = 0.01$ (compared with the control group).

Table IV. Effects of tobacco in DEN-treated ICRC mice

Treatment schedule	No. of animals	No. of tumor bearing animals	Mean no. of papillomas/ tumor-bearing animal ± SE
DEN[a] + 5% tobacco	9	9	21.7 ± 0.812[c]
DEN[a] + normal diet	8	8	4.6 ± 0.375
DEN[b] + 5% tobacco	10	10	3.4 ± 0.231
DEN[b] + normal diet	9	0	–
5% tobacco only	10	0	–

[a]DEN (4 mg/kg/day) was given through drinking water for 2 months continuously, followed by tobacco or normal diet.
[b]DEN (4 mg/kg/day) was given through drinking water for 2 weeks continuously, followed by tobacco or normal diet.
[c]$P < 0.001$ (compared with corresponding control group).

Discussion

The ICRC mouse is perhaps the only strain that shows megaesophagus, a condition which is seen in some strains of dogs (11), rats (12) and cats (13). It is a genetic disorder controlled by a recessive gene and is due to a disturbance of the nerve plexus in the lower end, resulting in impediment of passage of food, and consequent dilatation (7,14). Its pathogenesis is similar to megacolon.

DEN has been used as a carcinogen in different species of animals. Depending on the dose and route of administration, it affects different target organs (15). Even in the same species there are strain variations. Liver, lungs and esophagus are the main target tissues in rodents, and low dose favours induction of esophageal tumors (16). However, in studies conducted so far, tumor development takes at least 6–8 months, even on feeding of high doses. Clapp and Craig (17), fed a cumulative dose of 924 mg/kg body weight of DEN, spread over 22 weeks, to 10-week-old RF mice. No tumors were observed at the end of the treatment. However, in the following 2 months, 4% animals developed esophageal cancer. Similarly, in the experiments of Takayama and Oota (18), the minimum induction time for forestomach papillomas in DEN-fed ICR and C3H mice, was found to be 7–8 months. One of the important features of this study is that papillomas consistently develop in 100% ICRC mice, in a very short period of 12 weeks. The esophagus is the only affected organ. No tumors are seen in other organs. Further, tumors are produced, even at a very low cumulative dose of 28 mg/kg body weight. Also, continuous feeding of the carcinogen is not essential to induce esophageal tumors. Development of papillomas is dose dependent and increases with increasing duration of DEN feeding. In a recent study similar conclusions have been drawn by Rubio (19). Like Reuber and Lee (20), we also observed a higher incidence of tumors in younger animals. Our data indicates, that the ICRC mouse is a good model to study DEN-induced esophageal tumorigenesis.

Esophageal mucosa in the ICRC mouse is hyperplastic. This may be the main reason why only esophageal tumors are produced with such consistency in a relatively short exposure to the carcinogen. Clinical, as well as experimental studies, indicate that increased cell proliferation promotes tumorigenesis (21–23). In man, there are several instances where high cell turnover is associated with increased cancer risk in the gastrointestinal tract (21). High cell turnover has been observed in colonic mucosa in multiple polyposis, sporadic adenomas and ulcerative colitis (24–26). These conditions predispose to cancer. The esophageal mucosa of 'normal' subjects from China's Linxian county, which has a very high prevalence of esophageal cancer, shows higher cell turnover, particularly in the upper cell layers, when compared with the esophageal mucosa of a similar person from Jiaoxian county, which is a low incidence area (27). According to Lipkin (21), increased cell turnover could be used as a biomarker of increased susceptibility to cancer in the gastrointestinal tract.

The significance of the development of papilloma in the pathogenesis of esophageal carcinoma, needs to be investigated. In rats given *N*-methyl-*N*-nitrosaniline, Napalkov and Pozhavisski (28) demonstrated conclusively that carcinomas developed from papillomas. Stinson *et al.* (29) also showed development of papillary carcinomas from papillomas in rats treated with *N*-methyl-*N*-benzylnitrosamine. In this study we generally observed papillomas, occasionally some of them exhibited microinvasion, but frank infiltrative cancer was rarely seen. The observation period in this study is rather short (~6–8 months),

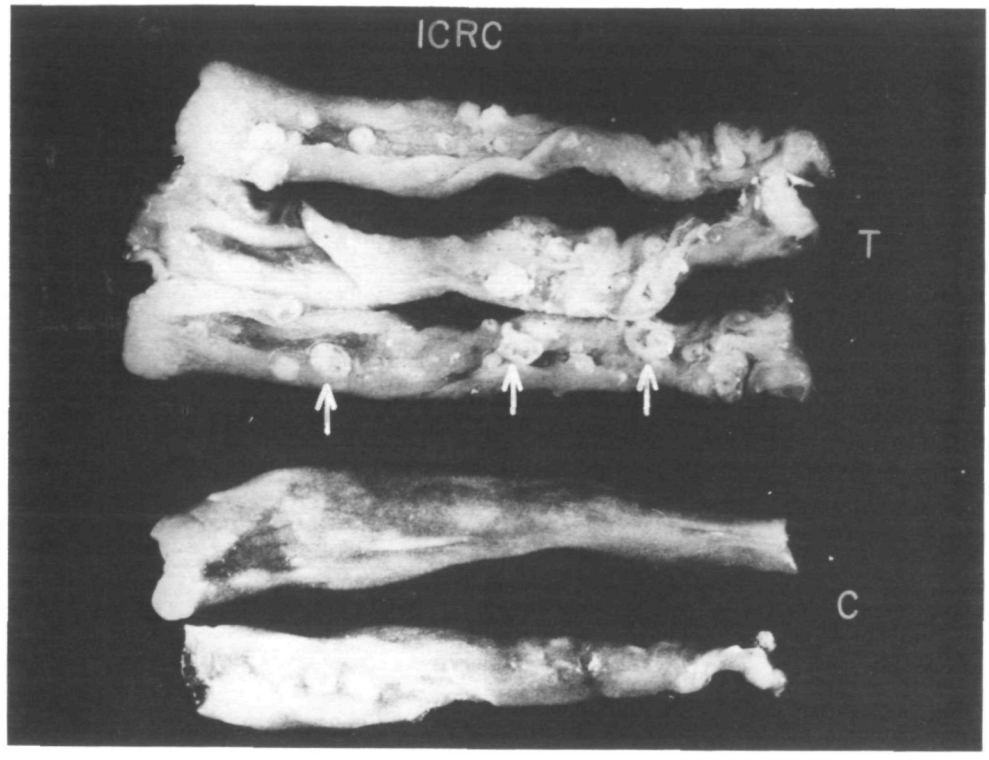

Fig. 6. Gross appearance of ICRC mouse esophagus. (T) 4-month DEN-treated esophagus showing large papillomas, many with ulceration (arrows). (C) Control esophagus of the same age animals, without any DEN treatment.

mainly because the animals become moribund after this period as a consequence of the mega-esophagus resulting in food stasis and aspiration pneumonia. This perhaps might be the reason why we did not observe frank malignancy.

Epidemiological studies indicate that, in man, chewing of tobacco increases the risk of cancer of the oral cavity and esophagus (10,30). The precise role of tobacco whether it is a carcinogen, promoter or both is not certain (31). Tobacco-specific nitrosamines given in drinking water, have also been reported to cause esophageal tumors in rats (32,33). On the other hand, Bock *et al.* (34) observed that the alkaline extract of unburned tobacco acts as a promoter. In the study there was a 5-fold increase in tumor incidence when tobacco was fed to DEN-primed animals. Our results are in concordance with Bock *et al.* (34), showing that tobacco principally acts as a promoter. Since the observation period in this study is rather short, no conclusions could be drawn about the carcinogenic potential of tobacco.

Other interesting features of this study are that tumor initiation could be induced by exposure to a low dose of DEN *in utero*, and tumors developed only in animals post-natally fed tobacco which, as mentioned above, is predominantly a promoter in our system. Animals fed tobacco alone post-natally did not develop tumors. Recently, Napalkov *et al.* (9) have observed a similar phenomenon in progeny of the mice fed dimethylbenzanthracine during pregnancy. The incidence of tumors in animals painted with 12-*O*-tetradecanoylphorbol-13-acetate during post-natal life was higher when compared to those exposed to only the carcinogen *in utero*. In their study, however, both the groups developed tumors and the promoter enhanced tumor growth. On the other hand, in our model, tumors developed in the F_1 only when they were fed a diet containing tobacco, at the low DEN dose employed by us. The ICRC mouse appears, therefore, to be an excellent model to study transplacental initiation and post-natal promotion of esophageal carcinogenesis.

To our knowledge, there is no natural model to simulate human tumors associated with tobacco chewing. Those who chew tobacco, keep the bolus in the mouth for a very long period of time (35). Tumors frequently occur at the site of contact of the bolus with the oral mucosa. The ICRC mouse with a mega-esophagus provides a good model. In these animals, because of the slowing down of the passage of food, tobacco remains in contact with the mucosa for a longer time. Also, no force feeding is resorted to, and tobacco is taken freely by the animals. Attempts have been made earlier to use the cheek pouch of the hamster, but without much success (36). As the ICRC mouse shows a large number of esophageal papillomas in a comparatively shorter period of carcinogen exposure, it provides a suitable model to study two-stage carcinogenesis. Our studies indicate that, the ICRC mice could be used to study the effect of food ingredients, e.g. capsaicin and other spices consumed daily in the food of the Indian population, as promoters of esophageal tumorigenesis. Further, the protective action of certain chemicals, e.g. retinoic acid, could also be tested, by studying inhibition of papilloma formation, on using the drug in conjunction with known carcinogens/promoters or both.

Nitrosamines, which are present widely in nature, have been strongly incriminated as human carcinogens (37). The question about what should be their 'permissible' limits for man is yet to be solved. Animal data are often used to fix the 'permissible' limits. Different species of animals are fed varying doses, for life time, which in the case of rodents, is ~2 years (38). The level at which no tumors are produced, is used to fix human

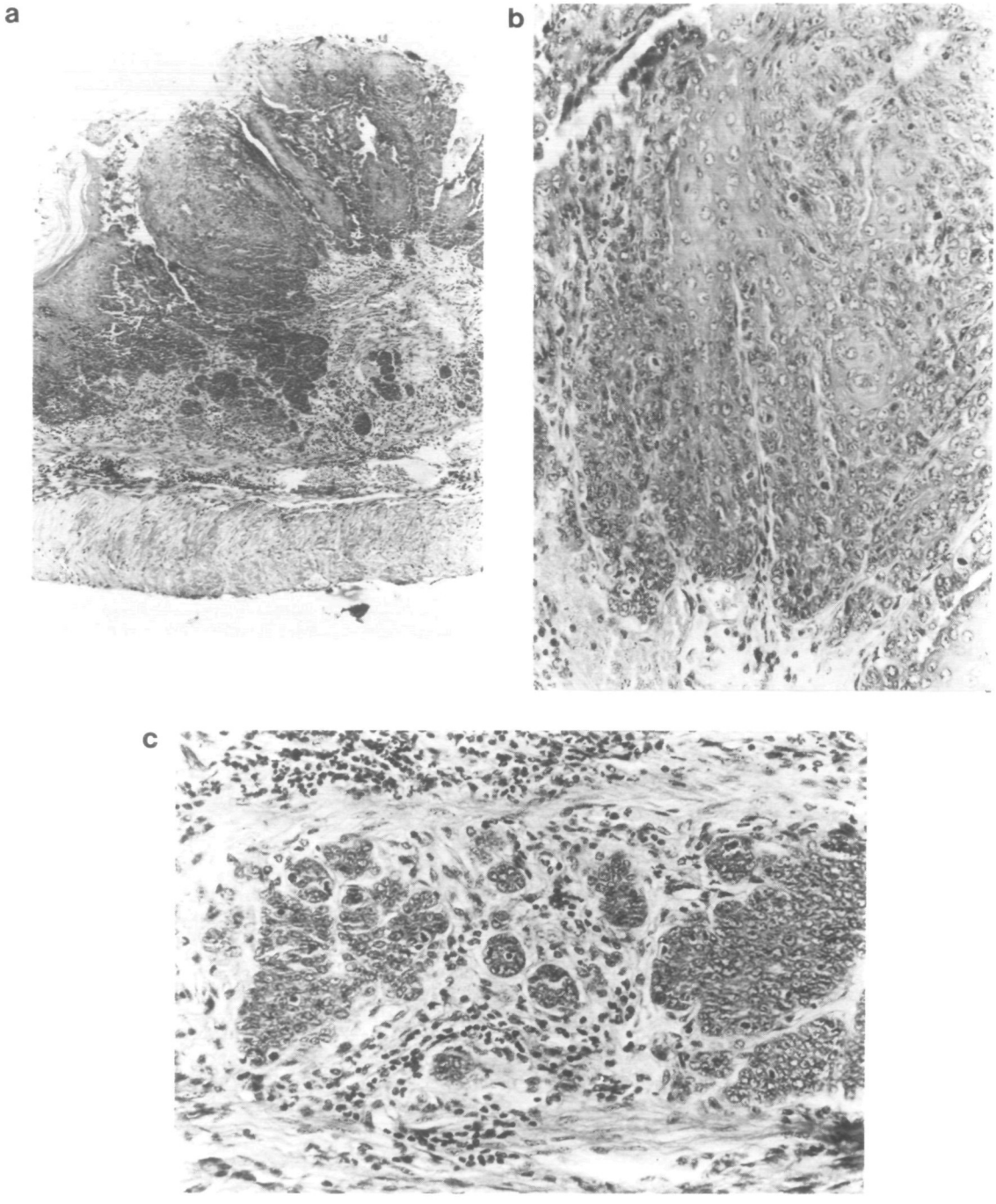

Fig. 7. (a) Low-power view of esophageal papilloma of ICRC mouse showing islands of cells infiltrating the underlying tissue (H&E; ×60). (b) High-power view of the papilloma showing a well-differentiated area in which the basal cell layer is intact and the cells are normally oriented (H&E; ×240). (c) High-power view of a portion of the papilloma showing groups of cells infiltrating into the deeper tissue. The tumor cells also show a large number of mitoses (H&E; ×240).

'permissible' limits. Lijinsky *et al.* (39) found that in adult Fischer rats, a cumulative dose as low as 1.35 mg/animal could induce tumors. In this study, tumors have been observed with a cumulative dose of 28 mg/kg body weight or 0.87 mg/ICRC mouse, considering that the average weight of the animals was 31 g. At this dose no tumors were seen at 3 months, but when the DEN-primed animals were fed a tobacco diet, tumors developed in 20% of the animals during the same period (data not shown). These observations suggest that, in future, all experiments aimed to determine 'permissible' levels, should employ a two-stage protocol of initiation and promotion. Only the dose at which no tumors are induced, even in the presence of a promoter, should be considered as the 'no-effect' or 'safe' level of a carcinogen.

Acknowledgement

The authors acknowledge Mrs Perin Notani for her advice in statistical analysis of the data.

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Received on March 22, 1989; revised on June 21, 1989; accepted on June 22, 1989