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Comparison of Reversibility of Rat Forestomach Lesions Induced by Genotoxic and Non-genotoxic Carcinogens

Masataka Kagawa, Kazuo Hakoi, Atsushi Yamamoto, Mitsuru Futakuchi and Masao Hirose[1]

First Department of Pathology, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467

Reversibility of forestomach lesions induced by genotoxic and non-genotoxic carcinogens was compared histopathologically. Groups of 30 to 33 male F344 rats were given dietary 0.1% 8-nitroquinoline, dietary 0.4–0.2% 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, an intragastric dose of 20 mg/kg body weight N-methyl-N′-nitro-N-nitrosoguanidine once a week, or 20 ppm N-methylnitrosourethane in the drinking water as a genotoxic carcinogen, or 2% butylated hydroxyanisole, 2% caffeic acid, 2% sesamol or 2% 4-methoxyphenol in the diet as a non-genotoxic carcinogen for 24 weeks. Ten or 11 rats in each group were killed at week 24. Half of the remainder were maintained on basal diet alone for an additional 24 weeks and the other half were given the same chemical for 48 weeks, and then killed. Forestomach lesions induced by genotoxic carcinogens did not regress after removal of carcinogens. In contrast, simple or papillary hyperplasia (SPH), but not basal cell hyperplasia (BCH), induced by non-genotoxic carcinogens clearly regressed after cessation of insult. SPH labeling indices in the non-genotoxic carcinogen-treated cases decreased after removal of the carcinogenic stimulus whereas BCH values were low irrespective of treatment. Atypical hyperplasia (AH), observed at high incidences in rats treated with genotoxic carcinogens, was also evident in animals receiving non-genotoxic agents, even after their withdrawal, albeit at low incidences. AH labeling indices remained high even without continued insult. These results indicate that even with non-genotoxic carcinogens, heritable alterations at the DNA level could occur during strong cell proliferation and result in AH development. This putative preneoplastic lesion might then progress to produce carcinomas.

Key words: Reversibility — Forestomach — Genotoxic carcinogen — Non-genotoxic carcinogen — Antioxidant

Recently, various phenolic compounds, i.e., butylated hydroxyanisole (BHA), caffeic acid (CA), sesamol, catechol, 4-methoxyphenol (4-MP), 4-methylcatechol and hydroquinone, were shown in our laboratory to have carcinogenic potential.[1-8] Of these compounds, BHA, CA, sesamol, 4-MP and 4-methylcatechol induce strong cell proliferation as well as cytotoxicity in the forestomach, with subsequent tumor development in both male and female rats.[1-11]

The mechanisms by which these phenolic compounds induce forestomach carcinomas are not fully understood; the compounds are generally considered to be non-genotoxic, even inhibiting carcinogen-induced mutagenesis.[12, 13] Binding of BHA or its metabolite(s) to forestomach DNA is under the limit of detection[14] and this agent did not show any clear initiating activity in a two-stage model of forestomach carcinogenesis in rats.[15] In contrast, BHA, CA and 4-methylcatechol strongly enhanced the second stage of rat forestomach carcinogenesis after initiation with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), N-methylnitrosourea (MNU), or N-dibutylnitrosamine (DBN).[7, 16-24] Therefore, these carcinogens may be considered to possess very weak initiating and very strong promoting activity. Generally, it requires more than one year of exposure to a high dose level for induction of carcinomas by these agents. It has been shown that forestomach hyperplasias and papillomas in rats receiving continuous oral treatment with 2% BHA for 12 to 72 weeks rapidly regress after cessation of the stimulus.[25-27] Similarly, urinary bladder hyperplasias induced by uracil, and liver foci induced by peroxisome proliferators, other so-called non-genotoxic carcinogens, have been reported to disappear after withdrawal of the chemicals.[28-33]

The situation appears different with established genotoxic carcinogens. 8-Nitroquinoline (8-NQ), 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2), MNNG and N-methylnitrosourethane (MNUR) all interact with forestomach DNA by alkylating DNA bases or forming DNA adducts. In the case of MNNG, a single intragastric administration induces forestomach carcinomas in rats.[7, 16, 20, 24] Therefore, the forestomach lesions induced by genotoxic carcinogens are not considered to be reversible. In the present experiment, the characteristics and reversibility of rat forestomach lesions induced by both genotoxic and non-genotoxic carcinogens were

[1] To whom all correspondence should be addressed.

compared histopathologically and for proliferative potential to assess their relevance to neoplasia.

MATERIALS AND METHODS

Animals Five-week-old male F344 rats were purchased from Charles River Japan Inc., Atsugi, randomly divided into 27 groups of 10 or 11 rats each and housed five or six to a plastic cage with wood chips for bedding in an air-conditioned room at a temperature of 24±2°C with a 12 h light-12 h dark cycle. They were given Oriental MF diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum* throughout.

Chemicals BHA (purity >98%) and 4-MP (purity >98%) were obtained from Wako Pure Chemical Industries, Osaka. CA (purity >98%), MNNG and MNUR were purchased from Tokyo Kasei Kogyo, Co., Tokyo. Sesamol (purity >98%) was from Fluka Chemie, AG, Switzerland, AF-2 (purity >98%) was from Ueno Pharmaceutical Co., Osaka, 8-NQ (purity >98%) was from Nakalai Tesque, Inc., Kyoto, and bromodeoxyuridine (BrdU) was from Sigma Chemical Co., MO, USA.

Experimental protocol At 6 weeks of age, groups of 30–33 rats were treated with 0.1% 8-NQ in the diet, 0.4% AF-2 in the diet, an intragastric dose of 20 mg/kg body weight MNNG once a week, 20 ppm MNUR in the drinking water, 2% BHA in the diet, 2% CA in the diet, 2% sesamol in the diet or 2% 4-MP in the diet for 24 weeks. In the case of AF-2, the dose was decreased to 0.2% 3 weeks after the beginning of experiment. Ten or 11 rats each were killed under ether anesthesia at the end of week 24. Another 10 or 11 rats each were maintained on basal diet alone for an additional 24 weeks, while the remaining animals received the same chemicals for the same period, and were then killed. Groups of 10 control rats were given basal diet alone for 24 and 48 weeks. Rats were weighed once every 2–4 weeks, and moribund animals were killed for autopsy. Five rats in each group received an intraperitoneal injection of 40 mg BrdU in saline one hour before being killed. The liver, kidneys and stomach were removed, then the liver and kidneys were weighed and 10% buffered formalin solution was injected into the stomach. Six sections each were cut from the anterior and posterior walls of the forestomach

Table I. Incidences of Hyperplastic Forestomach Lesions

Chemical	Treatment (weeks)		No. of rats	Simple or papillary hyperplasia (%)			Basal cell hyperplasia (%)			Atypical hyperplasia (%)
	Chemical	Basal diet		Mild	Moderate	Severe	Mild	Moderate	Severe	
8-NQ	24	0	10	5 (100)	0	0	0	0	0	0
	24	24	10	10 (100)	7 (70)**	0	6 (60)*	1 (10)	0	0
	48	0	11	11 (100)	9 (82)	0	10 (91)	2 (18)	0	1 (9)
AF-2	24	0	10	6 (60)	2 (20)	0	2 (20)	0	0	0
	24	24	8	8 (100)	5 (63)	0	6 (75)*	0	0	0
	48	0	11	11 (100)	6 (55)	0	10 (91)	0	0	0
MNNG	24	0	8	8 (100)	8 (100)	8 (100)	8 (100)	6 (75)	0	6 (75)
	24	24	6	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	5 (83)**	6 (100)
	48	0	6	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)
MNUR	24	0	10	10 (100)	10 (100)	10 (100)	6 (60)	3 (30)	0	2 (20)
	24	24	10	10 (100)	10 (100)	10 (100)	10 (100)*	8 (80)*	2 (20)	8 (80)*
	48	0	11	11 (100)	11 (100)	11 (100)	11 (100)	11 (100)	6 (55)	9 (82)
BHA	24	0	9	9 (100)	9 (100)	9 (100)	9 (100)	8 (89)	0	0
	24	24	10	10 (100)	4 (40)**	0***	10 (100)	4 (40)*	0	0
	48	0	11	11 (100)	11 (100)	11 (100)	11 (100)	11 (100)	6 (55)	0
CA	24	0	9	9 (100)	9 (100)	9 (100)	7 (78)	1 (11)	0	0
	24	24	10	10 (100)	2 (20)***	0***	6 (60)	0	0	1 (10)
	48	0	10	10 (100)	10 (100)	10 (100)	10 (100)	8 (80)	0	0
Sesamol	24	0	10	10 (100)	10 (100)	2 (20)	7 (70)	2 (20)	0	0
	24	24	10	10 (100)	4 (40)**	0	8 (80)	3 (30)	0	1 (10)
	48	0	11	11 (100)	11 (100)	1 (9)	11 (100)	11 (100)	0	2 (18)
4-MP	24	0	10	10 (100)	10 (100)	10 (100)	7 (70)	1 (10)	0	0
	24	24	10	7 (70)	0***	0***	6 (60)	0	0	1 (10)
	48	0	11	11 (100)	10 (91)	1 (9)	11 (100)	11 (100)	0	0
Control	0	24	10	3 (30)	0	0	0	0	0	0
	0	48	10	0	0	0	0	0	0	0

*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$ as compared with the respective groups treated with chemicals for 24 weeks and then killed.

and four from the glandular stomach. Tissues were routinely processed for hematoxylin-eosin (H & E) and anti BrdU immunohistochemical stainings.

For analysis of the labeling index (LI), counts were made of numbers of labeled cells in up to 600 epithelial cells of forestomach epithelium where hyperplasia (simple or papillary, basal cell and atypical) was most pronounced, and of carcinomas. LI values were expressed as numbers of labeled cells per 100 epithelial cells.

Student's *t* test and Fisher's exact probability test were used for statistical evaluation of the data.

RESULTS

Final body weights of animals treated with chemicals for 24 weeks were 2.4% (8-NQ) to 25.3% (AF-2) lower than the control value. However, decrease in body weight gain improved after cessation of chemical treatment to give final reductions of from 1.4% (CA) to 13.1% (AF-2). In animals treated with chemicals continuously for 48 weeks, the reduction was more prominent, i.e., 8.9% (8-NQ) to 34.7% (AF-2).

Lesions in the forestomach epithelium were classified into hyperplasias, papillomas and squamous cell carcinomas. Hyperplasias were divided into three categories: simple or papillary hyperplasias (SPH), respectively, presenting as a diffuse and flat upward growth of the squamous epithelium or diffuse papillary mucosal thickening with fine connective tissue stalks: basal cell hyperplasia (BCH) demonstrating downward basal cell growth. These lesions were further divided into mild (<0.1 mm), moderate (0.1–0.5 mm) and severe (>0.5 mm) grades depending on the thickness of the epithelium. Atypical hyperplasia (AH) was defined as areas of hyperplasia consisting of basal cell-like atypical cells with large nuclei and prominent nucleoli with loss of polarity in their arrangement. Intracellular cornification was sometimes observed. Papillomas presented as focal papillary or polypous lesions, with upward papillary or downward solid proliferation and increased connective tissue. Carcinomas were observed as atypical mucosal

Table II. Incidences of Forestomah Tumors

Chemical	Treatment (weeks)		No. of rats	Papilloma (%)	Carcinoma (%)
	Chemical	Basal diet			
8-NQ	24	0	10	0	0
	24	24	10	1 (10)	0
	48	0	11	6 (55)	0
AF-2	24	0	10	0	0
	24	24	8	0	0
	48	0	11	1 (9)	0
MNNG	24	0	8	8 (100)	2 (25)
	24	24	6	4 (67)	6 (100)**
	48	0	6	5 (83)	6 (100)
MNUR	24	0	10	5 (50)	0
	24	24	10	10 (100)*	4 (40)*
	48	0	11	11 (100)	9 (82)
BHA	24	0	9	0	0
	24	24	10	0	0
	48	0	11	1 (9)	1 (9)
CA	24	0	9	1 (11)	0
	24	24	10	0	0
	48	0	10	3 (30)	0
Sesamol	24	0	10	0	0
	24	24	10	0	0
	48	0	11	0	0
4-MP	24	0	10	0	0
	24	24	10	0	0
	48	0	11	0	0
Control	0	24	10	0	0
	0	48	10	0	0

*: $P<0.05$, **: $P<0.01$ as compared with the respective groups treated with chemicals for 24 weeks and then killed.

lesions often showing invasive growth into the connective tissue.

Incidences of forestomach lesions are summarized in Tables I and II. In rats treated with 8-NQ for 24 weeks, only 5 of 10 rats had mild SPH. However, 24 weeks after cessation of the treatment, 7 and 6 of 10 rats had moderate SPH and mild BCH, respectively. The degree of SPH and BCH development did not increase after continuous treatment for 48 weeks but papillomas were then found in 6 of 11 animals.

Treatment with AF-2 for 24 weeks induced moderate SPH in 2 and mild BCH in 2 of 10 rats, and the incidences of these lesions increased thereafter without further exposure. No additional increment was observed after continuous treatment with AF-2 for 48 weeks, and only one papilloma was found.

Continuous treatment with the strong forestomach carcinogen MNNG for 24 weeks induced SPH in all rats, AH in 6 of 8 rats, papillomas in all rats and carcinomas in 2 of 8 animals. At 24 weeks after cessation, all rats had AH and the incidences of carcinomas was significantly increased. All rats treated with MNNG for 48 weeks also had AH and carcinomas.

In the MNUR-treated groups, SPH (Fig. 1) was found in all rats. Incidences of mild and moderate BCH, and AH significantly increased after cessation of MNUR exposure. Papillomas were found in 5 of 10 rats after treatment for 24 weeks, and the incidence increased to 100% 4 of 10 rats had carcinomas by week 48 (Fig. 2). In rats treated with MNUR for 48 weeks, papillomas were found in all rats and carcinomas in 9 of 11 animals.

All and 8 of 9 rats treated with BHA for 24 weeks had severe SPH and moderate BCH, respectively, but after

Fig. 1. SPH in a rat receiving MNUR for 24 weeks. The epithelium demonstrates upward papillary proliferation with fine stromal connective tissue.

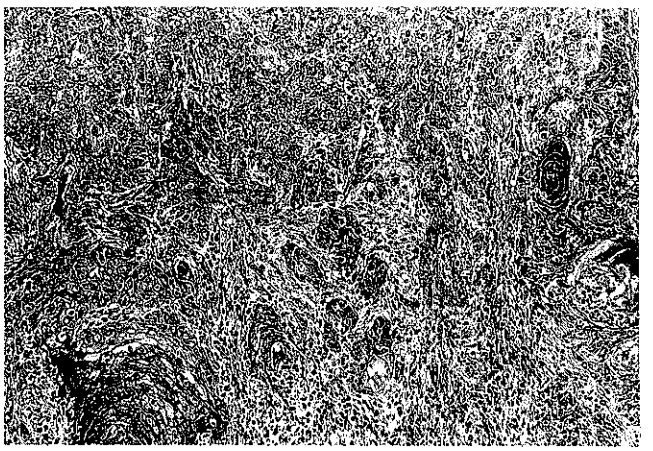

Fig. 2. Squamous cell carcinoma in a rat treated with MNUR for 24 weeks and then maintained on basal diet for a further 24 weeks. Atypical cell nests with cornification show downward invasive growth into the connective tissue.

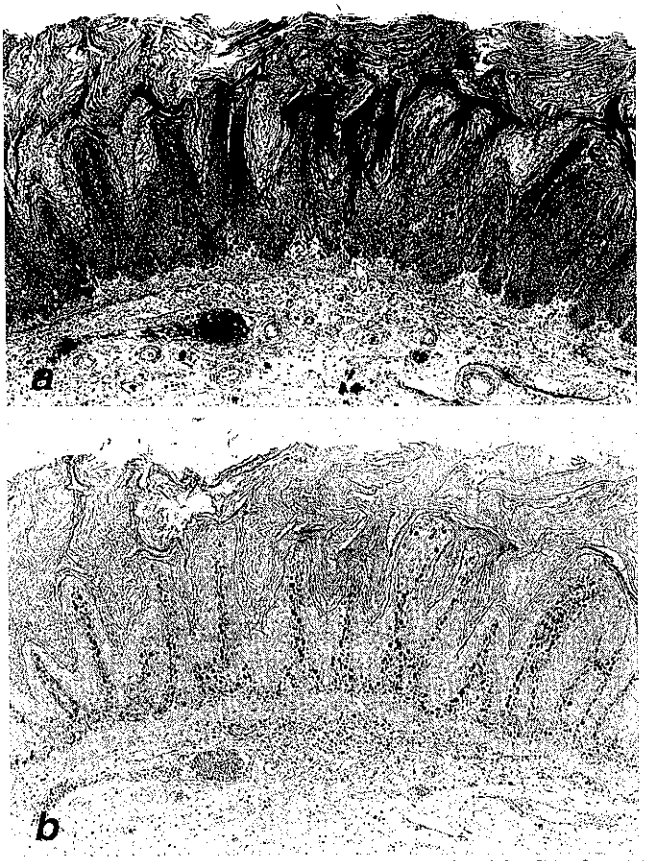

Fig. 3. (a) Severe SPH in a rat treated with CA for 24 weeks. Note the diffuse papillary mucosal thickening with thin connective tissue stroma. (b) Anti-BrdU immunohistochemical staining of the same lesion as shown in (a). Many cells in the basal layer are labeled with BrdU.

withdrawal of BHA, severe SPH disappeared and only 4 of 10 rats had moderate SPH by week 48. The incidence of moderate BCH also significantly decreased. After treatment with BHA for 48 weeks, all rats had severe SPH and 6 of 11 had severe BCH. In addition, one papilloma and one carcinoma each were found in this group.

Rats treated with CA showed a similar tendency to that seen with BHA, namely, all had severe SPH (Fig. 3a) at the 24 week time point, decreasing to 2 of 10 rats with moderate SPH 24 weeks later, when mild hyperplasia (Fig. 4a) predominated. The incidences of BCH did not decrease and one AH was found after cessation of CA treatment. All rats treated with CA for 48 weeks had severe SPH, and 8 of 10 rats had moderate BCH. Papillomas were found in one and three animals treated with CA for 24 weeks and 48 weeks, respectively.

In rats receiving sesamol for 24 weeks, all and 2 of 10 animals had moderate and severe SPH, respectively. After cessation of sesamol exposure, severe SPH disappeared and moderate SPH decreased to 40%. However, the incidences of mild and moderate BCH were not decreased. One AH (Fig. 5a) was found in this group. With continuous sesamol treatment for 48 weeks, severe SPH was induced in 1, moderate BCH in all and AH in 2 of 11 rats. No papillomas or carcinomas were found. In addition to hyperplasias, all rats treated with sesamol for 24 or 48 weeks demonstrated circular ulceration in the mid region.

All rats treated with 4-MP for 24 weeks had severe SPH (Fig. 6a) with circular ulceration in the mid region. However, 24 weeks after return to basal diet, only mild SPH was evident. The degree of BCH (Fig. 7a), in

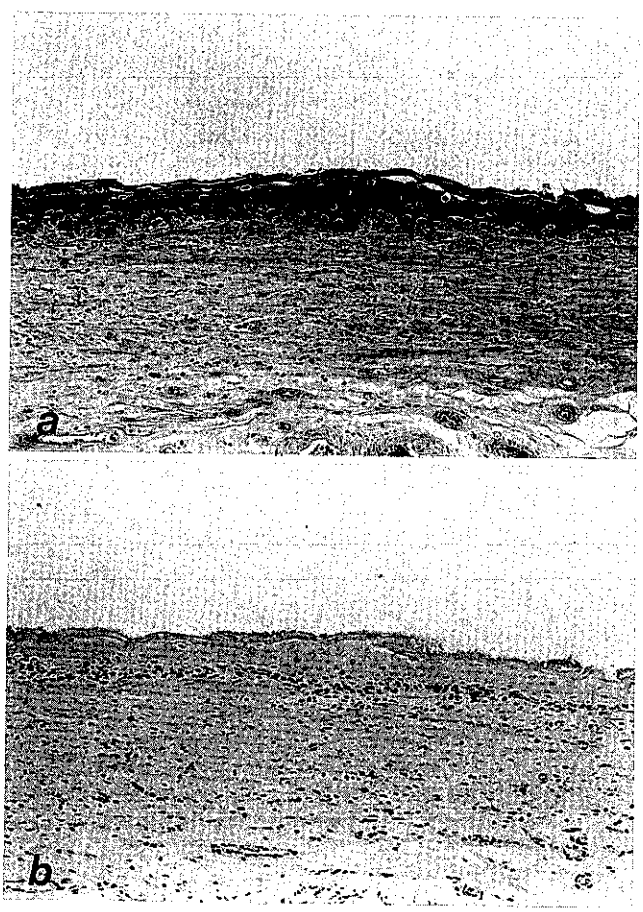

Fig. 4. (a) Mild SPH in a rat treated with CA for 24 weeks and then maintained on basal diet for 24 weeks. (b) Anti-BrdU immunohistochemical staining of the same lesion as shown in (a). Very few cells in the basal layer are labeled with BrdU.

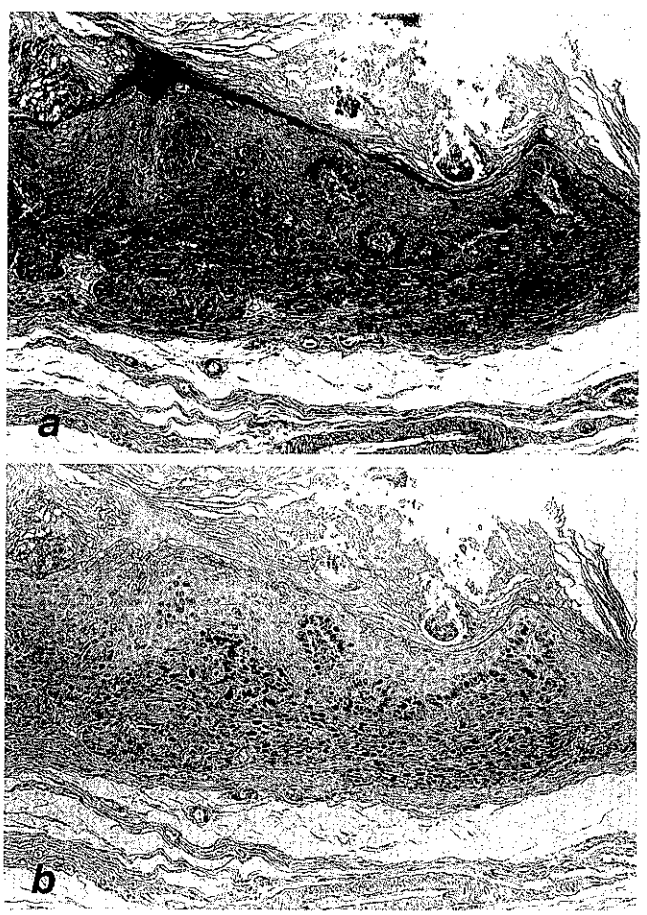

Fig. 5. (a) AH in a rat treated with sesamol for 24 weeks and then maintained on basal diet for 24 weeks. Note the downward growth of basal cell-like atypical cell nests with structural and nuclear irregularity. (b) Anti-BrdU immunohistochemical staining of the same lesion as shown in (a). Many labeled cells are present in atypical cell nests.

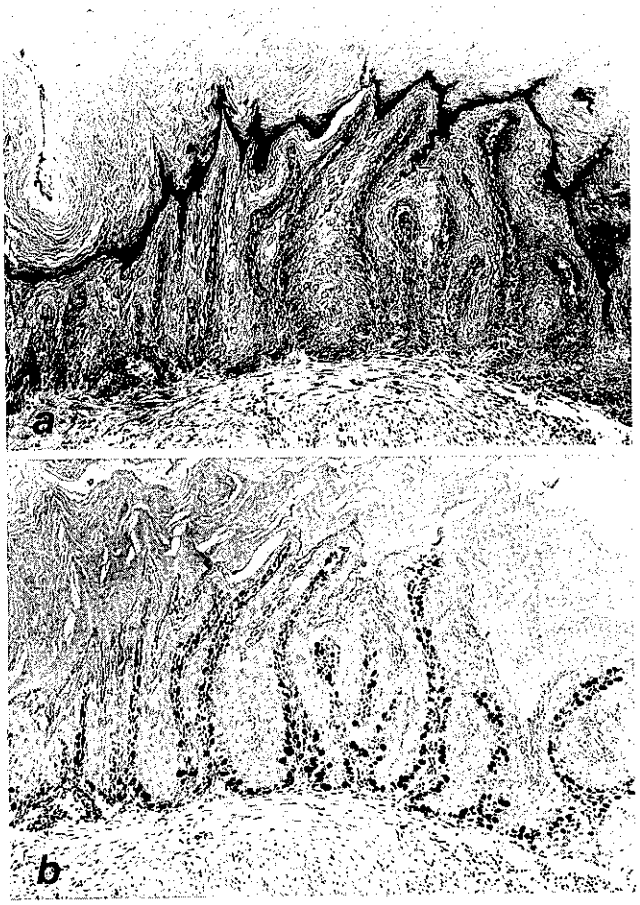

Fig. 6. (a) SPH in a rat treated with 4-MP for 24 weeks. The mucosa is diffusely thickened with upward papillary growth and fine connective tissue. (b) Anti-BrdU immunohistochemical staining of the same lesion as shown in (a). Many labeled cells are evident in the basal layer.

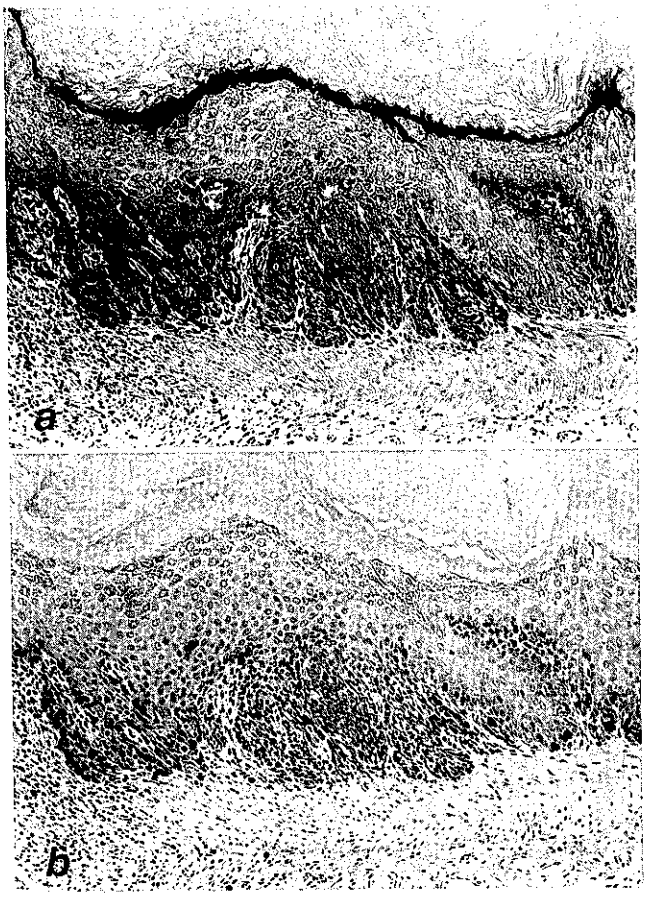

Fig. 7. (a) BCH in a rat treated with 4-MP for 24 weeks. Basal cells without atypia proliferate downward forming small nests at the center of this figure. (b) Anti-BrdU immunohistochemical staining of the same lesion as shown in (a). The number of labeled cells is smaller than in SPH.

contrast, did not decrease after cessation of 4-MP treatment. One rat had an AH in this group. In rats treated with 4-MP for 48 weeks, 10 and 1 of 11 animals had moderate and severe SPH, respectively, and all rats had moderate BCH. No rats had any papillomas or carcinomas.

LI data are summarized in Table III. The mild SPH in rats treated with 8-NQ or AF-2 demonstrated the same level, close to that of normal control epithelium, irrespective of the chemical discontinuation. However, in MNNG-treated groups, LI of the more severe SPH increased 24 weeks after MNNG treatment, with a further increment thereafter. On the other hand, in MNUR-treated groups, LI decreased after withdrawal of chemical treatment. LIs in SPH of rats treated with BHA, CA, sesamol or 4-MP were remarkably increased after treatment with 24 weeks (Figs. 3b and 6b) but considerably reduced 24 weeks thereafter (Fig. 4b). LI in BCH (Fig. 7b) remained lower than control values irrespective of chemical application and were not modified by further treatment with basal diet for 24 weeks. LI values for AH (Fig. 5b) ranged from 14.7 to 19.8, and those of squamous cell carcinomas in MNNG- and MNUR-treated groups were 14.7 and 27.0, respectively.

DISCUSSION

The present experiment clearly demonstrated that whereas the forestomach SPH induced by the non-genotoxic agents, BHA, CA, sesamol and 4-MP was reversible, forestomach hyperplasia induced by genotoxic 8-NQ, AF-2, MNNG and MNUR was not, rather developing into papillomas and squamous cell carcinomas after cessation of carcinogen treatment.

Table III. Labeling Indices of Hyperplasias and Carcinomas

Chemical	Treatment (weeks)		Simple or papillary hyperplasia	Basal cell hyperplasia	Atypical hyperplasia	Carcinoma
	Chemical	Basal diet				
8-NQ	24	0	11.6±1.5 (5)	—	—	—
	24	24	10.6±0.8 (5)	3.5±0.3 (3)	—	—
AF-2	24	0	10.2±1.6 (5)	—	—	—
	24	24	10.2±1.6 (5)	4.6±1.6 (4)	—	—
MNNG	24	0	21.1±2.8 (5)	2.3±0.5 (5)	16.9 (2)	—
	24	24	31.3±5.6 (5)**	2.4±0.4 (5)	16.1±6.2 (4)	14.7±7.0 (3)
MNUR	24	0	26.5±5.1 (5)	7.6±4.3 (3)	14.7 (1)	—
	24	24	19.2±3.5 (5)*	9.7±2.4 (3)	18.3 (2)	27.0±4.6 (4)
BHA	24	0	20.4±4.8 (5)	2.4±0.7 (5)	—	—
	24	24	11.6±1.9 (5)**	4.2±1.2 (5)	—	—
CA	24	0	27.8±3.6 (5)	1.2±1.2 (3)	—	—
	24	24	13.0±3.8 (5)***	0 (2)	14.0 (1)	—
Sesamol	24	0	25.3±6.3 (5)	7.1±2.5 (4)	—	—
	24	24	16.7±5.4 (5)*	5.4±2.1 (4)	19.8 (1)	—
4-MP	24	0	24.9±5.7 (5)	7.0±2.5 (3)	—	—
	24	24	13.4±1.7 (5)*	4.2±1.5 (3)	14.6 (1)	—

Values represent mean±SD.
( ): No. of rats. Control values in the normal lesions were 13.2±2.0 at week 24 and 7.2±1.1 at week 48.
*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$ as compared with the respective groups treated with chemicals for 24 weeks and then killed.

Similar reversibility in forestomach lesions associated with non-genotoxic carcinogens was earlier demonstrated for BHA,[25-27] ethyl acrylate[32] and methyl bromide.[34] In other organs, liver lesions induced by peroxisome proliferators,[30,31] and urinary bladder lesions induced by uracil,[28,29] the causative agents in both cases being non-genotoxic carcinogens, were similarly shown to be reversible.

MNNG and MNUR, both of which are well known as strong forestomach carcinogens, induced high incidences of AHs, without any regression after cessation of the carcinogen treatment. It is of interest that, although the incidences were low, similar lesions were also found in animals treated with CA, sesamol or 4-MP, even 24 weeks after removal of the chemical stimulus. LIs of AHs demonstrated relatively high levels regardless of the genotoxicity of inducing chemicals or discontinuation of exposure. Similar AH was observed in forestomach epithelium of hamsters treated with 2% BHA for 24–48 weeks and killed up to 48 weeks later.[35] Thus, AH may have autonomous growth potential. Recently, continuous oral treatment with 2% 4-MP for up to 104 weeks was demonstrated to induce forestomach squamous cell carcinomas at an incidence of 77% as well as a high incidence of AHs in male F344 rats, providing further evidence that the latter are preneoplastic forestomach lesions (unpublished data). Therefore, in the case of non-genotoxic forestomach carcinomas, it is possible that during strong cell proliferation, heritable alterations in DNA could have occurred, with the transformed cells developing into AHs and then carcinomas.

The degree of forestomach BCH induced by all genotoxic carcinogens increased, while BCH induced by CA, sesamol or 4-MP did not change, and BCH induced by BHA slightly decreased after withdrawal of chemicals. The LI values of BCH were lower than the normal epithelium control level irrespective of chemical treatment, suggesting that BCH has a generally low proliferative activity, as shown in a previous experiment,[33] and may be regarded as an unlikely preneoplastic forestomach lesion. However the irreversibility of BCH might reflect genotoxic potential of the chemicals. The lack of AHs and reversibility of BCH in BHA treated groups may thus reflect a weaker carcinogenic or DNA-transforming potential of this chemical than 4-MP, CA or sesamol, since the carcinogenic potential of BHA is weaker than that of 4-MP, CA or sesamol. In partial support of the possible weak *in vivo* genotoxicity of BHA, only small amounts of DNA adducts (i.e., 37.8 per 10^9 normal nucleotides) as evaluated by ^{32}P-post labeling assay were demonstrated in forestomach epithelium of rats treated with 2% BHA for 2 weeks.[36] In addition, any initiating activity of BHA for forestomach epithelium is very weak if present, since its prior administration for 24 weeks did not result in enhanced induction of forestomach tumors by subsequent MNNG or dibutylnitrosamine (DBN) exposure in rats.[15] On the other hand, combined treatment with genotoxic carcinogens such

as methylnitrosourea, 2,2′-dihydroxy-di-*n*-propylnitrosamine (DHPN) or 3,2′-dimethyl-4-aminobiphenyl (DMAB) during non-genotoxic BHA treatment caused a pronounced increase in the incidences of forestomach papillomas or carcinomas as compared with values for individual genotoxic carcinogen given alone.[37] It seems probable that epithelial cells are particularly susceptible to carcinogens during continuous strong proliferation. Similar findings were observed in the urinary bladder of rats simultaneously treated with the genotoxic carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) and non-genotoxic carcinogen uracil. The multiplicity of urinary bladder carcinomas was markedly greater with a combination of 3% uracil and 0.005% BBN as compared with the yields for the individual treatments.[38] Some BHA metabolites such as *tert*-butylhydroquinone, 3-*tert*-butyl-4,5-dihydroxyanisole, *tert*-butylquinone and 3-*tert*-butylanisole-4,5-quinone induce DNA damage in the forestomach epithelium after oral administration. Such genotoxic BHA metabolites are present in the forestomach epithelium or feces of treated rats, although at very low concentrations.[39,40] CA also causes metal-dependent DNA damage through H_2O_2 formation *in vitro*.[41] In addition, mutagenic compounds could be formed in the stomach by interaction of amines and nitrite,[42] or nitrite and phenolic compounds,[36,43,44] both of the latter being commonly present in the diet. Thus, it is possible that during strong cell proliferation, small amounts of genotoxic compounds such as hydroquinone metabolites, quinone metabolites, active oxygen species or food-derived mutagens interact with forestomach DNA and result in forestomach cell transformation even with "so-called" non-genotoxic forestomach carcinogens.

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