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Inhibition by β-Carotene of Upper Respiratory Tumorigenesis in Hamsters Receiving Diethylnitrosamine Followed by Cigarette Smoke Exposure

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In recent intervention studies, β-carotene failed to reduce or even increased the incidence of lung cancers in smokers. In the present investigation, the modifying effects of β-carotene at various doses on the development of upper respiratory tract tumors were investigated in Syrian hamsters treated with diethylnitrosamine (DEN) and cigarette smoke. A total of 120 male 5-week-old hamsters were divided into 4 groups, each consisting of 30 animals. After a single subcutaneous (s.c.) injection of 100 mg/kg DEN, hamsters in groups 1–4 were respectively administered diets supplemented with β-carotene at doses of 0.5%, 0.05%, 0.005% or 0% during experimental weeks 1 to 13, and simultaneously exposed to cigarette smoke. The duration of cigarette smoke exposure was 9 min twice a day, 5 days a week. Because of a marked reduction of body weight in group 1, the highest dose of β-carotene was changed to 0.25% after 10 days. In all groups, epithelial hyperplasias and/or papillomas were induced in the larynx and trachea. However, the incidence and multiplicity of papillomas in group 1 were significantly ($P<0.05$) lower than the group 4 values. Moreover, the β-carotene treatments significantly ($P<0.05$ or 0.01) reduced both the incidence and multiplicity of hyperplasias in a dose-dependent manner. The levels of retinol and β-carotene in the serum, and the retinol level in the liver, were also elevated with dose dependence. Our results thus indicate that β-carotene inhibits tumorigenesis, even at the high dose of 0.25%, under the present experimental conditions.

Key words: β-Carotene — Cigarette smoke — Respiratory tumorigenesis — Diethylnitrosamine — Hamster

Epidemiological studies have revealed an inverse association between vitamin A intake and risk of cancer at several sites, such as the lung, urinary bladder and larynx.[1)] Experimentally, vitamin A deficiency generally increases susceptibility to chemically induced neoplasia and an increased intake of the vitamin appears to protect against carcinogenesis in most cases.[1)] In fact, vitamin A has been shown to inhibit chemically induced neoplasia of the mammary gland, urinary bladder, skin and lung.[1)] β-Carotene, a naturally occurring provitamin A carotenoid, is one of more than 600 carotenoids that occur in nature, major sources being green-leaf vegetables and colored fruits.[2)] Early epidemiological findings suggested that a higher intake of β-carotene might be associated with a reduced risk of lung cancer,[3)] with consumption of vegetables rich in this agent providing particular protection.[1, 3)] The proposed mechanisms include antimutagenicity, inhibition of oxidation, inactivation of free radicals and immunological enhancement.[3–8)]

Experimental studies have demonstrated with a high degree of consistency that β-carotene inhibits lesion growth which is induced and promoted by a variety of carcinogens at various stages of carcinogenesis.[9–11)] For example, the preneoplastic foci induced by diethylnitrosamine (DEN) in the resistant hepatocyte model in rats have been reported to be significantly reduced by β-carotene.[12)] Prevention of 2-amino-3-methylimidazo[4,5-*f*]-quinoline-induced rat hepatocarcinogenesis in the initiation phase has also been described.[13)] However, there may be organ dependence and in fact, recent intervention studies have suggested that β-carotene may increase the incidence of lung cancers in continuing smokers.[14–16)]

It is well documented that cigarette smoking is closely associated with increased risk of cancers in various organs such as the lung, larynx, oral cavity, esophagus, oropharynx, pancreas, renal pelvis, urinary bladder and stomach.[17–19)] Tobacco-specific nitrosamines have been identified as abundant and strong carcinogens in chewing tobacco, snuff, tobacco-containing betel quid and tobacco smoke, being formed by *N*-nitrosation of nicotine during processing and storage.[20, 21)] We recently found that ciga-

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rette smoking exerts promoting effects on upper respiratory tract tumorigenesis in hamsters initiated with DEN, with squamous cell tumors (a common type of smoking-related lung tumor) being induced.[22] In the present study, the modifying effects of β-carotene at various doses on the development of upper respiratory tract tumors were investigated in Syrian hamsters to elucidate whether higher doses might enhance the tumorigenicity.

MATERIALS AND METHODS

Animals and chemicals A total of 120 male Syrian hamsters (Japan SLC, Inc., Shizuoka), 5 weeks old and weighing about 80 g at the commencement, were used in the experiment. The animals were housed, five per polycarbonate cage, in an air-conditioned room at 23±2°C with 60±5% humidity under a daily cycle of alternating 12-h periods of light and darkness. Oriental MF powder diet (Oriental Yeast Co., Ltd., Tokyo) and tap water were available *ad libitum*. DEN was obtained from Wako Pure Chemical Ind. Co., Ltd. (Osaka), β-carotene was kindly supplied by Roche Japan Inc. (Tokyo) and non-filter cigarettes were purchased from Japan Tobacco Co., Ltd. (Tokyo). The original β-carotene fluid suspension contained 30% β-carotene and 0.7% α-tocopherol suspended in 69.3% corn oil. It was confirmed by high-performance liquid chromatography (HPLC) that 91.6% of β-carotene was all-*trans* form.

Experimental protocol As schematically illustrated in Fig. 1, groups 1–4, each consisting of 30 hamsters, received a single subcutaneous (s.c.) injection of 100 mg/kg body weight of DEN at the commencement as an initiation treatment. Then all groups (1–4 groups) were exposed to smoke from non-filter cigarettes in a Hamburg type II smoking machine (Heinr. Borgwaldt, Hamburg, Germany) from weeks 1 to 13. The animals were continuously fed diet supplemented with 0.5% (group 1), 0.05% (group 2), 0.005% (group 3) or 0% (group 4) β-carotene during the post-initiation stage (weeks 1–13). These diets were prepared every 3–4 days based on stability data, with no significant reduction in β-carotene levels being noted in the remaining diet even after 7 days (data not shown).

All animals were exposed to smoke from 30 cigarettes, twice a day, 5 days a week. The smoke was generated under standard conditions (puff volume, 35 ml; puff duration, 2 s; puff frequency, 1/min). Each puff was diluted seven times with fresh air before reaching the exposure chamber. For each exposure, the animals were separately placed in animal tubes with their muzzles protruding into the inhalation chamber and exposed continuously for 9 min to the smoke. Each cigarette contained 2.7 mg of nicotine, and the tar content was 28 mg. Body weight for each animal was recorded weekly.

After the completion of the treatment at 12 weeks, all surviving animals were killed and examined. The heart, spleen, liver, adrenals, kidneys and testes of each animal were weighed. Moribund or dead animals were also completely autopsied for histological examination. The respiratory tract was fixed by intratracheal instillation of a 10% neutral-buffered formalin solution. At macroscopic examination, the mucosal surface of the respiratory tract was painted with Wright's eosin methylene blue (Omron, Tokyo), and then carefully examined using a stereoscopic microscope for counting focal/nodular or papillary lesions.

Two or three sections each from the upper, middle and lower thirds of the trachea, one midsagittal section of the left lung, and one section each from the three right lobes of the lung along the axes of the airways were embedded in paraffin, sectioned and stained with hematoxylin and eosin for light microscopic examination.

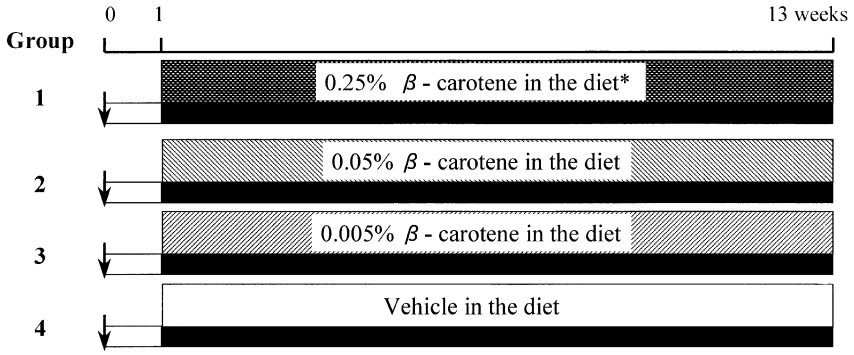

Fig. 1. Experimental design. ↓ diethylnitrosamine (DEN) 100 mg/5 ml/kg b.w., s.c., ■ cigarette smoke (9 min, 2 times/day, 5 days/week), * started at a dose of 0.5% and changed to 0.25% after 10 days.

Liver tissues and serum samples from 5 animals of each group were stored at −40°C for measuring retinol, β-carotene (*trans*) and β-carotene (*cis*) by HPLC.

The results were statistically analyzed by means of analysis of variance (ANOVA) and Fisher's exact probability test.

RESULTS

The body weight curves of hamsters (Fig. 2) showed a marked reduction of both body weight (13.7% down) and food consumption (67% down) in group 1 after 7 days, in comparison with the group 4 values, and the highest dose of β-carotene was therefore changed to 0.25% after 10 days. There were no significant differences in the final body weights among groups 1, 2 and 4, whereas the values for group 3 were significantly ($P<0.05$) higher by 10% than in group 4. Data for total intake of β-carotene estimated from the food consumption data are given in Table I. There were no significant differences in diet intake among groups 1, 2, 3 and 4. The values of mean daily intake of β-carotene in groups 1–3 (calculated relative to mean body weight values) were 10.7, 2.45 and 0.22 g/kg body weight, respectively. Thus, daily and total intakes of β-carotene were clearly dose-related. There were no differences in the relative organ weights with the exception of that for the adrenals in group 1, which was significantly ($P<0.05$) lower than the group 4 value.

At autopsy, papillary or nodular lesions ranging from 1 to 3 mm in diameter and/or smaller white spots or patches were observed in the larynx and trachea in all the groups. Macroscopically, focal or nodular lesions suggesting epithelial hyperplasias showed irregular surfaces and simple thickening, and papillary lesions suggesting papillomas showed a cauliflower-like pattern with a coarse granular surface. The incidence of papillary lesions in group 1 (14.3%) was significantly ($P<0.05$) reduced as compared with that in group 4 (46.4%). Similarly, the

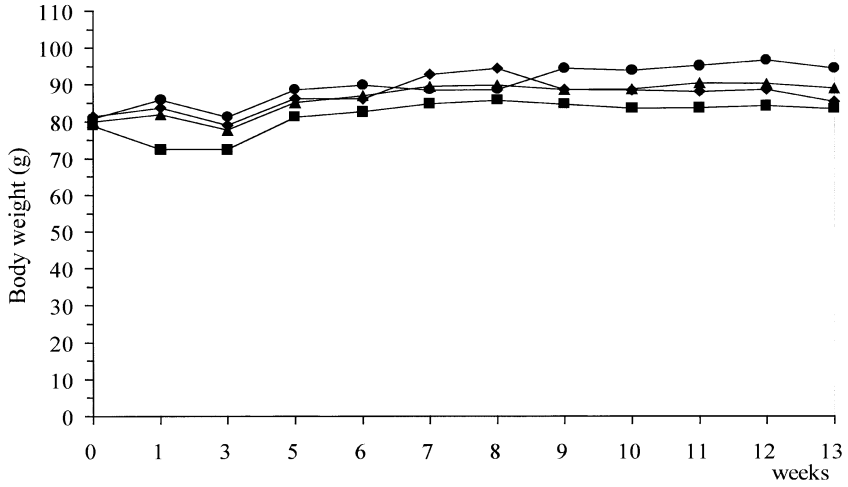

Fig. 2. Body weight curves of hamsters. ■ group 1, ▲ group 2, ● group 3, ◆ group 4.

Table I. Food Consumption and Intake of β-Carotene

Group	Food consumption (g/hamster/day)	β-Carotene	
		Daily intake (mg/kg/day)	Total intake (g/kg)
1. DEN→β-carotene (0.5→0.25%)	4.25	127.53	10.71
2. DEN→β-carotene (0.05%)	5.19	29.20	2.45
3. DEN→β-carotene (0.005%)	5.01	2.65	0.22
4. DEN alone	4.77	—	—

Table II. Number of Macroscopic Proliferative Lesions in the Larynx/trachea of Hamsters Treated with DEN Followed by Cigarette Smoke Exposure and β-Carotene

Group	No. of animals examined	Incidence (%)			Number of proliferative lesions per hamster (mean±SD)		
		Papillary	Focal/nodular	Total	Papillary	Focal/nodular	Total
1. DEN→β-carotene (0.5→0.25%)	26	4 (14.3)*	14 (53.8)**	15 (57.6)**	0.16±0.37*	1.08±1.18**	1.24±1.30**
2. DEN→β-carotene (0.05%)	29	11 (37.9)	21 (72.4)*	23 (79.3)	0.41±0.56	1.24±1.05**	1.65±1.26**
3. DEN→β-carotene (0.005%)	28	10 (35.7)	21 (75.0)*	23 (82.1)	0.42±0.63	1.35±1.16*	1.78±1.34*
4. DEN alone	28	13 (46.4)	27 (96.4)	28 (100)	0.46±0.50	2.14±1.14	2.60±1.31

∗ Significantly different from group 4 at $P<0.05$. ∗∗ Significantly different from group 4 at $P<0.01$.

Table III. Distribution of Macroscopic Proliferative Lesions in the Larynx/trachea of Hamsters Treated with DEN Followed by Cigarette Smoke Exposure and β-Carotene

Group	Number of proliferative lesions per hamster (mean±SD)					
	I *a)*		II		III	
	Papillary	Focal/nodular	Papillary	Focal/nodular	Papillary	Focal/nodular
1. DEN→β-carotene (0.5→0.25%)	0.12±0.33	0.28±0.54*	0.04±0.20	0.40±0.65*	0*	0.36±0.70
2. DEN→β-carotene (0.05%)	0.24±0.44	0.24±0.51*	0.14±0.44	0.69±0.81	0.03±0.19	0.31±0.60
3. DEN→β-carotene (0.005%)	0.11±0.31	0.39±0.57	0.29±0.53	0.57±0.79	0.04±0.19	0.39±0.63
4. DEN alone	0.18±0.39	0.86±1.01	0.11±0.31	0.82±0.55	0.18±0.39	0.32±0.55

a) I, larynx and upper trachea; II, middle trachea; III, lower trachea.
∗ Significantly different from group 4 at $P<0.05$.

incidences of focal/nodular lesions in groups 1, 2 and 3 were significantly ($P<0.05$ or $P<0.01$) decreased as compared with that in group 4 (Table II). In addition, the combined incidences of papillary and focal/nodular lesions in group 1 (57.6%) was significantly lower than that in group 4 (100%) (Table II). The mean numbers of proliferative lesions in the larynx and trachea per animal, and the total numbers in each group are shown in Table II. The total numbers of papillary and focal/nodular lesions in groups 1, 2 and 3 were clearly smaller than in group 4. The multiplicity of papillary lesions in group 1 (0.16±0.37) was significantly ($P<0.05$) decreased as compared to the group 4 value (0.46±0.50), along with those of the focal/nodular lesions in groups 1, 2 and 3 ($P<0.05$). The multiplicities of papillary lesions plus focal/nodular lesions in groups 1, 2 and 3 were significantly ($P<0.01$ or $P<0.05$) smaller than in group 4. The inhibitory effects in terms of both incidences and multiplicities were exerted in a clear dose-dependent manner.

As shown in Table III, papillary and focal/nodular lesions were widely distributed in the larynx to the lower trachea, but their multiplicities were greater in the larynx and upper and middle trachea among these anatomical sites in group 4. The multiplicities of focal/nodular lesions of the larynx and, upper and middle trachea in group 1, and the larynx and upper trachea in group 2 were significantly ($P<0.05$) decreased as compared to the group 4 values, and that of papillary lesions of the lower trachea in group 1 was significantly ($P<0.05$) decreased as compared to the group 4 value.

Microscopically, proliferative lesions in the larynx and trachea were diagnosed as papillomas and hyperplasias. The mean numbers of such proliferative lesions in the larynx and trachea per animal are given in Table IV. The multiplicities of hyperplasias in groups 1 (0.65±1.01) and 2 (0.82±1.07) were significantly ($P<0.05$) decreased as compared to the group 4 value (1.64±1.78). That for papillomas in group 1 (0.26±0.66) was lower than, though

not statistically significantly different from, the group 4 value (0.53±0.88). The multiplicities of papillomas plus hyperplasias in groups 1 and 2 were 0.92±1.19 and 1.17±1.07, respectively, being significantly (P<0.01 or P<0.05) decreased as compared to that for group 4 (2.17±1.84). Thus, the β-carotene treatments significantly (P<0.05 or P<0.01) reduced the multiplicity of hyperplasias in a clear dose-dependent manner. Interestingly, the multiplicity of eosinophilic liver cell foci was significantly greater in group 1 than in group 4, although no liver tumors were observed (Table V).

HPLC analysis confirmed that the β-carotene treatment dose-dependently increased the levels of retinol and β-carotene in the serum, and that of retinol in the liver (Table VI), although these effects were not statistically significant, possibly because of small numbers of samples and relatively large standard deviations.

Table IV. Multiplicity of Histopathologically Confirmed Proliferative Lesions in the Larynx/trachea

Group	Number of proliferative lesions per hamster (mean±SD)		
	Papilloma	Hyperplasia	Total
1. DEN→β-carotene (0.5→0.25%)	0.26±0.66	0.65±1.01*	0.92±1.19**
2. DEN→β-carotene (0.05%)	0.34±0.48	0.82±1.07*	1.17±1.07*
3. DEN→β-carotene (0.005%)	0.53±0.96	1.25±1.17	1.78±1.61
4. DEN alone	0.53±0.88	1.64±1.78	2.17±1.84

* Significantly different from group 4 at P<0.05. ** Significantly different from group 4 at P<0.01.

DISCUSSION

Our results clearly indicate that β-carotene inhibits tumorigenesis even at a dose as high as 0.25% under the present experimental conditions, in contrast to recent epidemiological data indicating a lack of protection against development of lung cancers in smokers with 30–35 years smoking history in Finland,[14] colorectal adenomas in the USA[23] and second skin cancers in the USA.[24] In the Finnish study, the group receiving the combination of β-carotene plus retinol showed a 28% increase in lung cancer incidence.[15, 16] However, these trials may have been too short (5–7 years) in follow-up duration to allow evalu-

Table V. Liver, Kidney and Lung Lesions in Hamsters

	Group	1	2	3	4
	No. of animals	25	29	28	28
Liver	Basophilic focus	2.60±2.04[a]	2.10±2.44	1.25±2.59	1.50±2.21
	Eosinophilic focus	0.68±0.85*	0.06±0.25	0.14±0.44	0.25±0.70
	Clear cell focus	2.20±3.43	1.51±1.68	1.46±3.16	0.71±1.58
Kidney	Tubule basophilia	2.96±1.77	2.34±1.93	2.89±2.18	2.14±1.43
Lung	Bronchiolar hyperplasia	1.96±1.67	1.93±1.62	1.78±1.47	1.60±2.04
	Papilloma	0	0.03±0.18	0.03±0.18	0

a) Mean±SD.
* Significantly different from group 4 at P<0.05.

Table VI. Retinol Levels in the Serum and Liver Tissue of Hamsters

	Group	Retinol (μg/g)	β-Carotene (*trans*) (μg/g)	β-Carotene (*cis*) (μg/g)
Serum	1. DEN→β-carotene (0.5→0.25%)	0.42±0.03[a]	0	0
	2. DEN→β-carotene (0.05%)	0.41±0.03	0	0
	3. DEN→β-carotene (0.005%)	0.37±0.04	0	0
	4. DEN alone	0.32±0.05	0	0
Liver tissue	1. DEN→β-carotene (0.5→0.25%)	53.06±3.49	9.35±5.48	1.04±0.05
	2. DEN→β-carotene (0.05%)	50.30±2.68	4.39±1.45	0.79±0.34
	3. DEN→β-carotene (0.005%)	44.36±3.84	2.25±1.47	0.50±0.28
	4. DEN alone	37.43±4.89	0.21±0.05	0.06±0.01

a) Mean±SD.

ation of effects on the promotional stages of lung carcinogenesis. Such intervention studies may need a longer follow-up duration to confirm the long-term effects of β-carotene administration in lung carcinogenesis.[25]

Previously, we reported promotion by cigarette smoke of the development of proliferative lesions in the upper respiratory tract of hamsters initiated with DEN.[22] Cigarette smoke is a complex mixture containing many compounds which have been demonstrated to act as carcinogens, tumor promoters or cocarcinogens. Among them, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, formaldehyde and volatile phenols have been identified as active agents in the gas and particulate phases.[26] In addition, in our previous study, the malondialdehyde level of the lung was increased in the smoke-exposed group at weeks 2 and 12,[22] and similar increases in lipid peroxide (LPO) levels in the lung have been reported in rats exposed to cigarette smoke for 7 days,[27] suggesting that cigarette smoke-induced lung damage may be related to the production of free radicals. The lung contains non-protein sulfhydryl groups, radical scavengers such as vitamin E and superoxide dismutase, and antioxidant enzymes, including glutathione peroxidase and glutathione reductase, which metabolize lipid peroxides or inhibit their production in animal tissues.[28] Non-enzymatic antioxidants include vitamins C and E, and carotenoids.[29]

β-Carotene functions as an antioxidant in many, but not all, *in vitro* systems.[4] The antioxidant functions of carotenoids would be expected to neutralize free radicals or metabolic intermediates that are highly reactive since they contain non-paired electrons.[8] Such reactive species are capable of initiating lipid peroxidation by reacting with polyunsaturated fatty acids, inactivating proteins and enzymes by attacking amino acids, and damaging RNA and DNA by reaction with guanine.[25] Free radical species can result from photochemical reactions and oxidant stress, for example induced by cigarette smoking, but free radicals are also a result of normal cell metabolism.[29] If the cell is insufficiently protected by enzymatic and non-enzymatic antioxidants, free radicals can react with biomolecules and thus damage cellular structures.[25] In a recent experiment,[30] high dietary fat was found to enhance benign and malignant tumor development in the mouse lung, and this was partially prevented by β-carotene. Thus, the antioxidant action of β-carotene could be involved in the inhibitory effects on the development of upper respiratory tract tumors found in the present study. Similar effects on 8-hydroxydeoxyguanosine formation were noted, so that this could be one of factors underlying its beneficial influence.

On the other hand, it has been hypothesized that prooxidant interactions of free radicals and other oxidants in smoke with β-carotene may enhance procarcinogenic oxidative damage in the lung, although β-carotene functions most effectively as an antioxidant under the low oxygen tension found in many tissues.[31, 32] β-Carotene can react with radicals of gas-phase smoke to form radical adducts,[33] which may then react as second radicals to form non-radical products or with oxygen to form peroxyl radicals.[34] Therefore, the induction of rapid β-carotene autoxidation by smoke-borne oxidants in the relatively high-oxygen environment of the lung is hypothesized to amplify smoke-induced oxidative damage.[34] However, β-carotene autoxidation does not accelerate lipid peroxidation,[34] though the gas-phase smoke induces both lipid peroxidation and rapid β-carotene autoxidation. In the liposomal system, β-carotene thus behaves as a weak antioxidant rather than as a prooxidant. Further studies in cellular and *in vivo* systems should be directed at establishing the antioxidant and prooxidant consequences of β-carotene smoke interactions in living cells and animals.[34]

Experiments in animals have also suggested that β-carotene is able to reduce DNA and chromosomal damage induced by alkylating agents such as ethylnitrosourea[35] and methylmethanesulfonate,[36] and by procarcinogens such as benzo[*a*]pyrene[37] and cyclophosphamide.[38] It has been extensively investigated in cancer-chemopreventive studies, displaying suppressing activity against oral and colon tumors.[39, 40] One of the critical events in carcinogenesis is the formation of DNA adducts.[41] Improper DNA repair generates permanent changes via subsequent DNA replication, and vitamin A and β-carotene have been shown to prevent or to decrease DNA damage caused by chemical carcinogens, as detected directly by measuring the level of DNA adducts[42, 43] or indirectly by measuring sister-chromatid exchanges,[44] chromosome aberration[38, 44] or DNA repair.[45, 46] In general, the inhibition of DNA-damage induction by vitamin A is ascribed to an effect on the metabolism of carcinogenic compounds, resulting in a decreased amount of ultimate carcinogen.[41] These effects of β-carotene may also play a role in the chemopreventive action.

In conclusion, our results indicate that β-carotene exerts inhibitory effects even at as high a dose as 0.25%, under the present experimental conditions, although the precise mechanism involved remains to be investigated.

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