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Lack of Modifying Effects of Intratracheal Instillation of Quartz or Dextran Sulfate Sodium (DSS) in Drinking Water on Lung Tumor Development Initiated with 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in Female A/J Mice

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Abstract: The purpose of the present study was to investigate the effects of inflammation, induced by intratracheal instillation (i.t.) of quartz as an environmental factor in the lung or drinking of dextran sulfate sodium (DSS) as an environmental factor in the colon on lung tumors in female A/J mice initiated with NNK. For comparison, colonic preneoplastic lesions, aberrant crypt foci (ACF), were also assessed. A/J mice at 6 weeks of age were divided into 5 groups, and Groups 1, 2 and 3 were pretreated with NNK (2 mg / 0.1 ml saline / mouse, intraperitoneal injection) at week 0. For a week, 2% DSS in drinking water was administered to the mice in Groups 2 and 4 beginning in week 1. In week 2, the mice of Groups 3 and 5 were exposed to intratracheal instillation of quartz (0.1 mg/rat) suspended in 25 μ l saline. The experiment was terminated after 16 weeks. The results for the lung tumors and colonic ACFs showed a lack of modifying effects of the inflammation in either site. Hematologically and histopathologically, the inflammation induced by 0.1 mg quartz in the lung and 2% DSS in the colon was lacking or only mild at the end of 16 weeks. These results suggest that there may be differences in sensitivity to inflammation that determine tumor promoting potential. (*J Toxicol Pathol* 2009; 22: 179–185)

Key words: NNK, lung tumor, quartz, DSS, inflammation, A/J mouse

Introduction

Lung cancer is common throughout the world and a leading cause of mortality in developed countries¹. While cigarette smoking is regarded as the primary cause, with cessation of smoking being the major target for reduction of incidence, the risk of development of lung cancer development remains elevated even after individuals quit smoking and exposure to environmental tobacco smoke from others continues to be a problem². Therefore, it is important to investigate the biological characteristics of lung tumors and the effects of environmental factors on lung tumorigenesis.

The tobacco-specific N-nitrosamine, 4-(Methylnitros-

amino)-1-(3-pyridyl)-1-butanone (NNK), conceivably plays an important role in tobacco-related human lung cancer given its strong potential to induce lung lesions in rodents³. However there are no reports concerning NNK and colonic tumors or colonic preneoplastic lesions, aberrant crypt foci (ACF). Previously, we demonstrated that treatment with 8-methoxypsoralen (8-MOP) during the initiation phase strongly inhibits the lung tumorigenesis induced by a single intraperitoneal (i.p.) injection of NNK in female A/J mice^{4,5}. CYP2A6 is recognized as being involved in the mutagenic activation of promutagens such as tobacco-specific N-nitrosamines⁶ and is also responsible for metabolism of 70%–80% of nicotine to the inactive metabolite cotinine in humans⁷. It has also been reported that 8-MOP inhibits CYP2A^{8,9} and we have previously demonstrated that 3-day intake of 100 ppm 8-MOP strongly reduces induction of lung tumors¹⁰. When adenocarcinomas induced by single i.p. injection of NNK in A/J mice for 52 weeks were used for a mutation analysis, high rates of change were noted in codons 12 and/or 61 of K-ras (positive in 21/23 lung tumors)¹¹. We have also focused on establishing a short-term bioassay

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model for identification of chemopreventive agents acting during the initiation phase of lung carcinogenesis¹². Examination of the time course of NNK-induced lung tumor development to determine the most appropriate and shortest period to assess the effects of test agents, with 8-MOP as a typical example, showed that two treatments with NNK and a 12-weeks duration are effective for detection of lung cancer chemoprevention¹².

There are many reports showing that inflammation promotes carcinogenic potential in various organs¹³. The mechanism of the promotion has also been reported to induce cytokines, inducible nitric oxide synthase (iNOS)¹⁴, cyclooxygenase-2 (COX-2)¹⁵, NF-kappa B¹⁶, tumor growth factor (TNF)^{13,17} and so on by inflammatory changes. If their markers are transported by the blood flow from the primary organ of the inflammation to other organs, the promotive potential may be induced in the organs that are distant from the original site of inflammation.

The purpose of the present study was to investigate the effects of inflammation, induced by intratracheal instillation (i.t.) of quartz as an environmental factor in the lung or drinking of dextran sulfate sodium (DSS) as an environmental factor in the colon, on lung tumors in female A/J mice initiated with NNK. For comparison, colonic preneoplastic lesions, aberrant crypt foci (ACF), were also assessed. Quartz and DSS are typical agents causing inflammation in the lung¹⁸ and the colon¹⁹, respectively. To assess the effect of the inflammation in the local organs, which were also the carcinogen-initiated organs, assessments of the effects of lung inflammation on lung tumor and colonic inflammation on ACF in the colon were performed. Furthermore, to assess the effect of the inflammation in the distant organs, which were not carcinogen-initiated organs, assessments of the effects of lung inflammation by quartz on ACF in the colon and colonic inflammation by DSS on lung tumor were also performed.

Materials and Methods

Chemicals

NNK was purchased from Toronto Research Chemicals (Toronto, ON, Canada), and DSS (molecular weight: 36000–50000 daltons) was purchased from MP Biomedicals Inc. (Burlingame, CA, USA); quartz dust (DQ-12) with a particle diameter of not more than 7 μm was obtained from Deutsche Montan Technologie (GmbH, Germany). Physiological saline (Otsuka Isotonic sodium chloride solution from Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) was used as the vehicle for both test substances.

Animals

Female A/J mice (5 weeks of age), purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained in the Division of Animal Experimentation, Life Science Research Center, Kagawa University, according to the Institutional Regulations for Animal

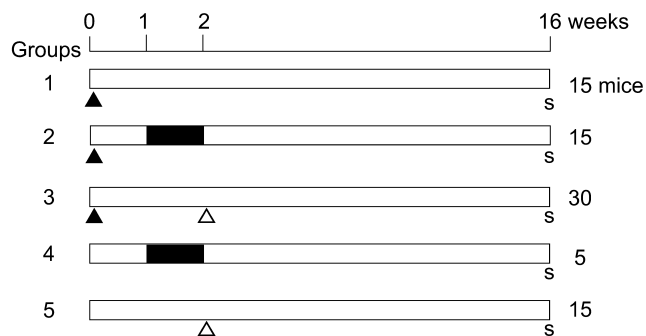


Fig. 1. Experimental design. A total of 80 A/J mice, were divided into 5 groups at 6 weeks of age. Groups 1, 2 and 3 were pretreated with NNK (▲: 2 mg / 0.1 ml saline / mouse i.p.) in week 0. The mice of Groups 2 and 4 received 2% DSS (■) in drinking water for one week from week 1. In weeks 2, the mice of Groups 3 and 5 were exposed to intratracheal instillation of quartz (△: 0.1 mg/rat) suspended in 25 ml saline. The experiment was terminated after 16 weeks (S: sacrifice).

Experiments. The protocols were approved by the Animal Care and Use Committee for Kagawa University. The animals were housed in polycarbonate cages with white wood chips for bedding, and given free access to drinking water and a basal diet, Oriental MF (Oriental Yeast Co., Ltd., Tokyo, Japan), under controlled conditions of humidity ($60 \pm 10\%$), lighting (12 h light/dark cycle) and temperature ($24 \pm 2^\circ\text{C}$). The experiments were started after a 1-week acclimation period.

Experimental design and tissue preparation

The design for the experiment is outlined in Fig. 1. A total of 80 mice at 6 weeks of age were divided into 5 groups of 15 (Groups 1, 2 and 5), 30 (Group 3) and 5 mice (Group 4). The mice of Groups 1, 2 and 3 were pretreated with NNK (2 mg / 0.1 ml saline / mouse i.p.) in week 0. In week 1, 2% DSS in drinking water was administered to the mice of Groups 2 and 4. In week 2, the mice of Groups 3 and 5 were exposed by i.t. to quartz (0.1 mg/rat) suspended in 25 μl saline using a specially designed aerolizer (Penn Century, Philadelphia, PA, USA) under deep anesthesia. The experiment was terminated after 16 weeks. All mice were weighed and then sacrificed by exsanguination after collection of blood samples to be processed for hematological examinations from the abdominal aorta under deep anesthesia. At autopsy, the lungs, colon, liver and kidneys were excised and weighed, infused with 10% neutral buffered formalin, and carefully inspected grossly. All macroscopically detected lung nodules were counted under a stereomicroscope, and each lung lobe was examined histopathologically. The colons were inflated with fixative, opened longitudinally, stained with 0.2% methylene blue in saline and scored under a light microscope for the number of ACFs²⁰. The lungs, liver and kidneys were then immersed in 10% neutral buffered formalin for a week, and slices were

Table 1. Body and Organ Weights of the Mice

Groups Treatment	1 NNK i.p.	2 NNK i.p.+DSS	3 NNK i.p.+quartz i.t.	4 DSS	5 Quartz i.t.
Number of mice	13	14	24	5	14
Body weight (g)	24.50 ± 1.67	22.83 ± 1.78 ^b	22.79 ± 1.97 ^c	26.53 ± 3.14	28.22 ± 3.43
Liver					
Absolute (mg)	1089 ± 56	954 ± 77 ^{a,b}	993 ± 96 ^c	1127 ± 96	1127 ± 199
Relative (%)	4.46 ± 0.26	4.18 0.21	4.36 0.29	4.27 ± 0.30	3.99 ± 0.43
Kidneys					
Right					
Absolute (mg)	211 ± 316	113 ± 10	118 ± 12	143 ± 9	139 ± 23
Relative (%)	0.86 ± 1.27	0.49 ± 0.03	0.52 ± 0.04	0.54 ± 0.05	0.49 ± 0.05
Left					
Absolute (mg)	125 ± 10	111 ± 9 ^b	119 ± 11 ^c	143 ± 14	133 ± 21
Relative (%)	0.51 ± 0.04	0.49 ± 0.01	0.52 ± 0.04 ^c	0.54 ± 0.04	0.47 ± 0.06
Lung					
Absolute (mg)	166 ± 12	171 ± 18	170 ± 20	177 ± 13	175 ± 25
Relative (%)	0.68 ± 0.07	0.75 ± 0.09	0.75 ± 0.06 ^c	0.67 ± 0.07	0.63 ± 0.10

^a P<0.05 vs group 1 by Tukey-Kramer test. ^b P<0.05 vs group 4. ^c P<0.05 vs group 5.

Table 2. Hematological Data of the Mice

Groups Treatment	1 NNK	2 NNK+DSS	3 NNK+quartz	4 DSS	5 Quartz
Number of mice	6	13	17	4	11
WBC	2583 ± 1037	2215 ± 585	1635 ± 956 ^a	2900 ± 1109	2445 ± 770
Hb	14.0 ± 0.3	14.5 ± 0.8	14.6 ± 0.5	15.1 ± 0.7	14.8 ± 0.7

^a P<0.05 vs. Group 4 by Tukey-Kramer test.

routinely processed for embedding in paraffin for histopathological examination of hematoxylin and eosin-stained sections and immunohistochemistry. In the hematological examinations, the red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT) and white blood cell count (WBC) were determined using routine laboratory procedures; the hematological examinations were performed by SRL, Inc. (Tokyo, Japan).

Statistical analysis

The incidences of ACFs in the colons were analyzed by the Kruskal-Wallis test. The body and organ weight data, hematological data, and the multiplicities of lung proliferative lesions and ACFs in the colon were analyzed by Tukey-Kramer test (multi-comparison test).

Results

A total of 10 mice died during the experimental period, seven (6 mice of Group 3 and 1 of Group 5) after intratracheal instillation of quartz, and the other 3 (2 mice of Group 1 and 1 of Group 2) spontaneously during the

experimental period. None of the groups demonstrated any remarkable changes in their general conditions.

The final body and organs weights are shown in Table 1. The body weights of the mice in Groups 2 and 3 were significantly decreased compared with those of the mice in Groups 4 and 5, respectively. The absolute weights of the liver and left kidneys of the mice in Groups 2 and 3 were also decreased compared with the mice in Groups 4 and 5, respectively, but the relative weights did not show any significant differences.

For the hematological studies, 9–17 mice per group were examined due to technical errors in blood collection. The data for WBC and Hb are summarized in Table 2. The WBCs of the mice in Group 3 (NNK+quartz) were significantly decreased compared with the Group 4 values (DSS). However, no significant intergroup differences were noted in Hb (shown in Table 2), RBC, HT, MCV, MCH and MCHC (data not shown).

Macroscopically, whitish nodules were detected in the lungs of the NNK-treated mice in Groups 1, 2 and 3; their multiplicities based on counting under a stereomicroscope are summarized in Table 3. Contrary to expectation, there were no significant intergroup differences. No significant macroscopic changes were observed in the liver, kidney or colon. ACFs were seen in some mice treated with NNK

Table 3. Macroscopical Nodules in the Lungs

Groups	Treatment	No.	Right	Left	Bilateral
1	NNK	13	2.2 ± 2.1	1.2 ± 1.8	3.4 ± 3.6
2	NNK+DSS	14	2.4 ± 1.7	1.4 ± 1.5	3.8 ± 2.8
3	NNK+quartz	24	1.8 ± 1.5	1.4 ± 1.6	3.2 ± 2.3
4	DSS	5	0.2 ± 0.4	0.0	0.2 ± 0.4
5	Quartz	14	0.0	0.2 ± 0.4	0.2 ± 0.4

There was no significant intergroup difference by Tukey-Kramer test.

Table 4. ACFs Analysis in the Colon

Groups	No.	Incidence (%)	No. of ACFs	No. of crypts	
1	NNK	13	38.5	0.62 ± 0.96	4.50 ± 2.78
2	NNK+DSS	14	35.7	0.50 ± 0.76	6.86 ± 2.34
3	NNK+quartz	24	20.8	0.25 ± 0.53	7.00 ± 2.45
4	DSS	5	0.0	0.00	0.00

Incidence: there was no significant intergroup difference by Kruskal-Wallis test. ACFs and crypts: there was no significant intergroup difference by Tukey-Kramer test.

(Groups 1–3), and the incidences and multiplicities are indicated in Table 4. There were no significant intergroup differences in these values or the number of crypts per ACF.

Histopathologically, evidence of inflammation in the lungs was only slight with intratracheal instillation of quartz in Groups 3 (Fig. 2-B) and 5 as compared with the NNK alone group, Group 1 (Fig. 2-A). DSS also induced only mild inflammatory changes in the colons of the mice in Group 2 (Fig. 2-D) and 4 compared with the NNK alone group, Group 1 (Fig. 2-C).

Discussion

In the present experiment, there were no significant modifying effects of the inflammation-inducing regimens in distant or local target organs of NNK carcinogenesis in the female A/J mice. Thus, the quantitative data for the lung tumors and colon ACFs showed no changes. Furthermore, hematologically and histopathologically, the inflammation induced by 0.1 mg quartz in the lung and 2% DSS in the colon was milder than expected. This suggests that female A/J mice may be relatively insensitive to these agents.

In quartz dust-exposed construction workers, obstructive and restrictive loss of lung function has been detected²¹, as well as chronic obstructive pulmonary disease (COPD)^{22,23}. These conditions are associated with an inflammatory cell response characterized by alveolitis with recruitment of inflammatory cells, particularly neutrophils, and may result in pulmonary fibrosis and impaired lung function²⁴. Intratracheal instillation of quartz into rats produces an inflammatory reaction followed by histological changes characteristic of lung fibrosis²⁵, which is similar to the above-noted human conditions. We have also employed

quartz as a positive control for material induced lung inflammation in F344 rats and have established a bioassay model by intratracheal instillation for detection of lung toxicity due to fine particles in F344 male rats^{18,26–28}. From these previous experiments in our laboratory, 0.1 mg quartz was concluded to be a sufficient dose to cause inflammation in the lungs of a mouse (estimated average body weight: 20 g) based on our previous findings for 1 mg quartz in F344 rats (estimated average body weight: 200 g)^{26,27}.

DSS is a synthetic sulfated polysaccharide well known to induce inflammatory changes in the colons of mice^{19,29}, rats³⁰, hamsters³¹ and guinea pigs^{32,33}. It has been reported that BALB/c mice develop acute colitis with signs of diarrhea, gross rectal bleeding, and weight loss within 6–10 days after ingesting 3%–10% DSS¹⁹. The most widely used inflammatory bowel disease model is that of mice given DSS, and Tanaka et al. reported that 1-week administration of 2% DSS after initiation with azoxymethane (AOM, 10 mg/kg body weight i.p.) in male ICR mice³⁴, or a single i.g. administration (200 mg/kg body weight) of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) or 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) in CD-1 mice³⁵, resulted in powerful promoting activity on colon carcinogenesis. This was associated with induction of beta-catenin, cyclooxygenase-2 and inducible nitric oxide synthase. In the present experiment, however, the dose of 2% DSS administration had no without major influence. This is in line with one report of mouse strain differences in the inflammatory responses of the colonic mucosa induced by dextran sulfate sodium and thus differential susceptibility to PhIP-induced large bowel carcinogenesis³⁶.

From the dose information of other reports and our previous experiments, both 0.1 mg quartz i.t. and

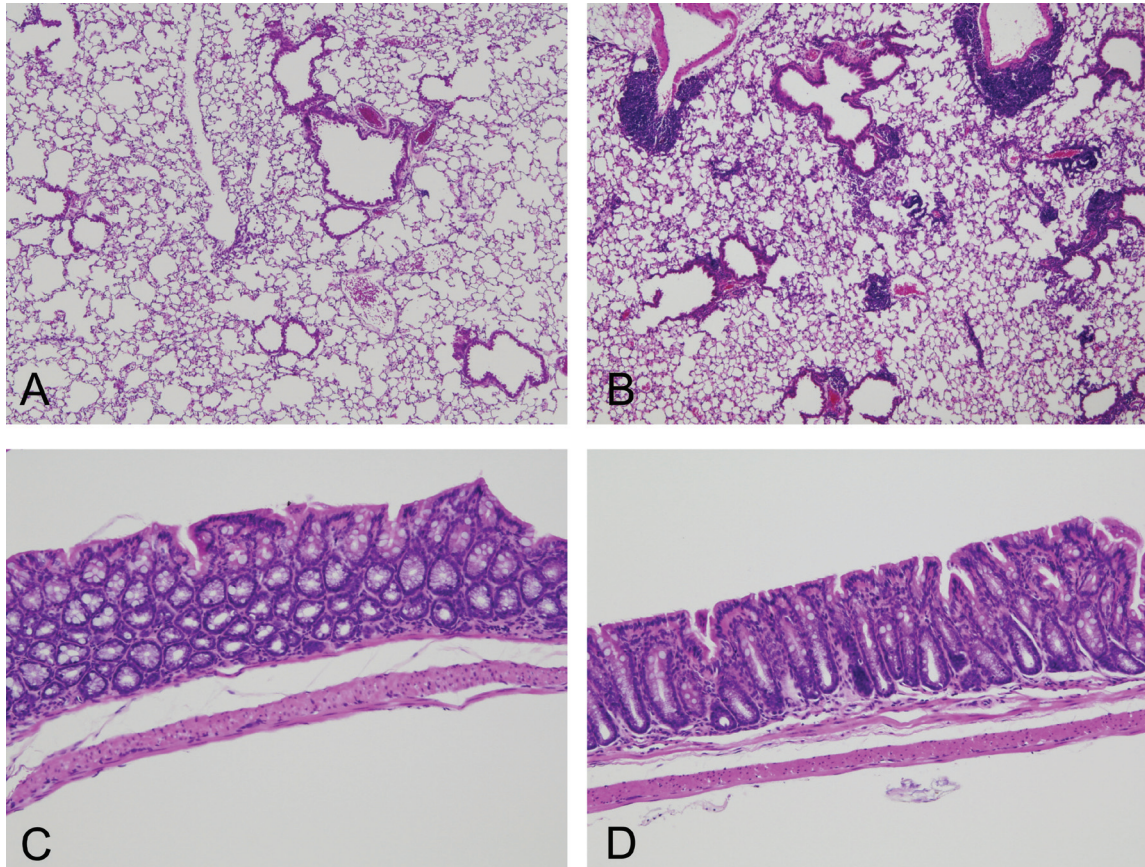


Fig. 2. Histopathological findings for the lungs and colons of the A/J mice. A, Group 1 lung (magnification: $\times 20$); B, Group 3 lung ($\times 20$); C, Group 1 colon ($\times 100$); D, Group 2 colon ($\times 100$). Histopathologically, a change was only noted for intratracheal instillation of quartz in Group 3 (Fig. 2–B). DSS also induced only mild inflammatory changes in the colons of Group 2 (Fig. 2–D).

administration of 2% DSS in drinking water were expected to induce inflammatory changes in each target organ in the present experiment, however, the results were contrary to our expectation. The results of our present experiment indicate that the A/J mouse may have high resistibility against treatments including an inflammatory reaction. Furthermore, there may be specific differences in inflammatory responses to quartz and DSS. An experiment is currently being performed to confirm this hypothesis.

In conclusion, the results of the present experiment showed a lack of modifying effects of inflammation-targeting regimens on the development of lung tumors and colonic ACFs initiated with NNK in female A/J mice indicative of species or strain differences in sensitivity to the agents employed. However the general tumor promoting effects of inflammation, assessment of alternative inducing regimens is warranted.

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