管の間の測定値のばらつきは10%以内で
あり、安定した測定結果が得られること
が分かった。吸入チャンバー内のクロル
ピリフォス濃度は、最低濃度である0.07
ppbでも測定が可能であり、今回の方法は
クロルピリフォスの極低濃度暴露実験で
吸入チャンバー内の濃度の把握に有効で
あった。

　以上のように、クロルピリフォスを被
験物質とし、室内濃度指針値である0.07
ppbを考慮した0.07、0.21および0.7 ppb
を目標暴露濃度として暴露技術の開発を
行ない、その実用性について検証した。
その結果、加熱・バブリング法により気
化させる方法がクロルピリフォスの極低
濃度暴露実験に利用できることを確認で
きた。

E．結論
　化学物質を極低濃度で実験動物に経気
道暴露するための技術開発を目的として、
クロルピリフォスを対象として室内濃度
指針値（0.07 ppb）を考慮した濃度で動
物に全身暴露する方法の開発を試みた。
その結果、加熱・バブリングする方法に
より0.07、0.21および0.7 ppbの目標暴露
濃度で吸入暴露する方法を開発すること
が出来た。

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G．知的財産権の出願・登録状況（予定を含む）

1．特許取得
なし

2．実用新案登録
なし

3．その他
なし

表 1　　吸入暴露装置の設定条件とチャンバー内のクロルピリフォス濃度

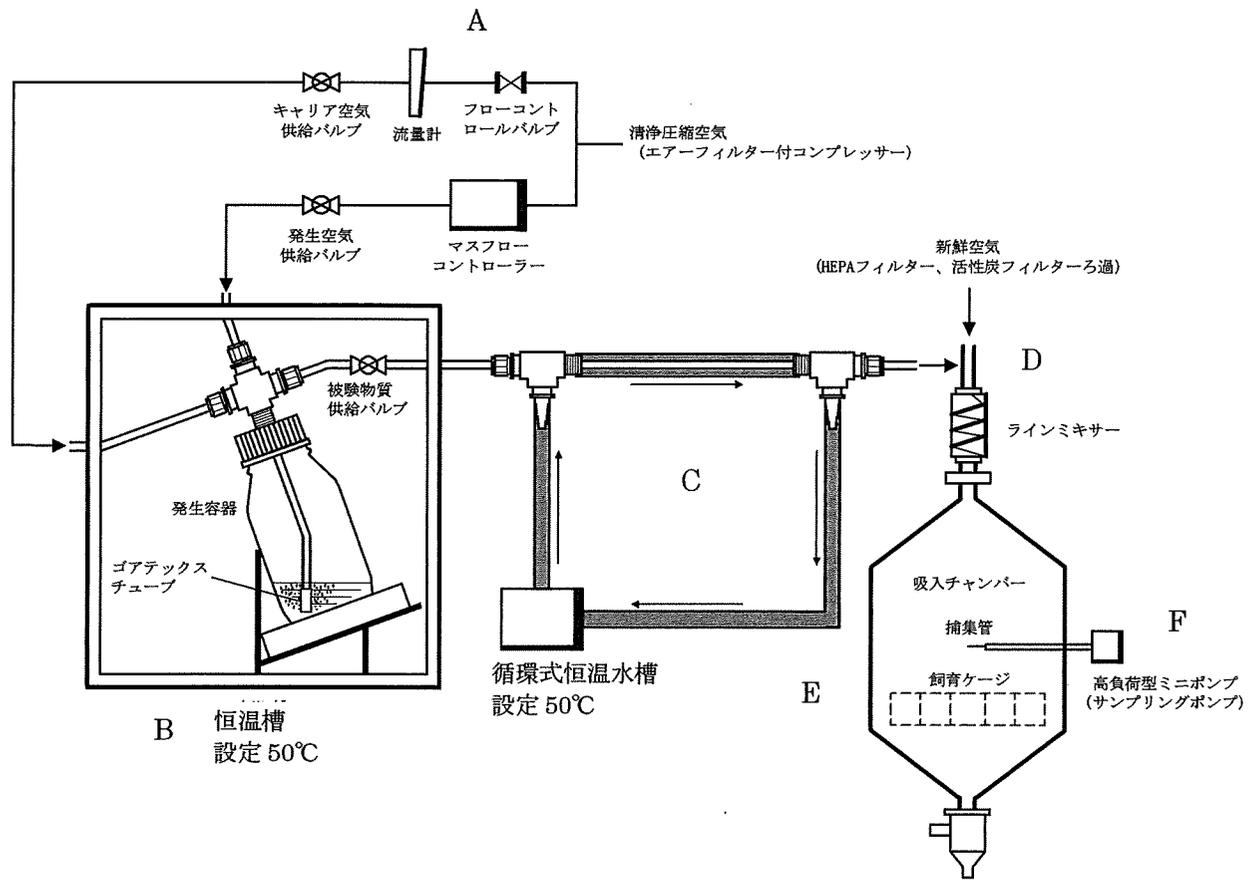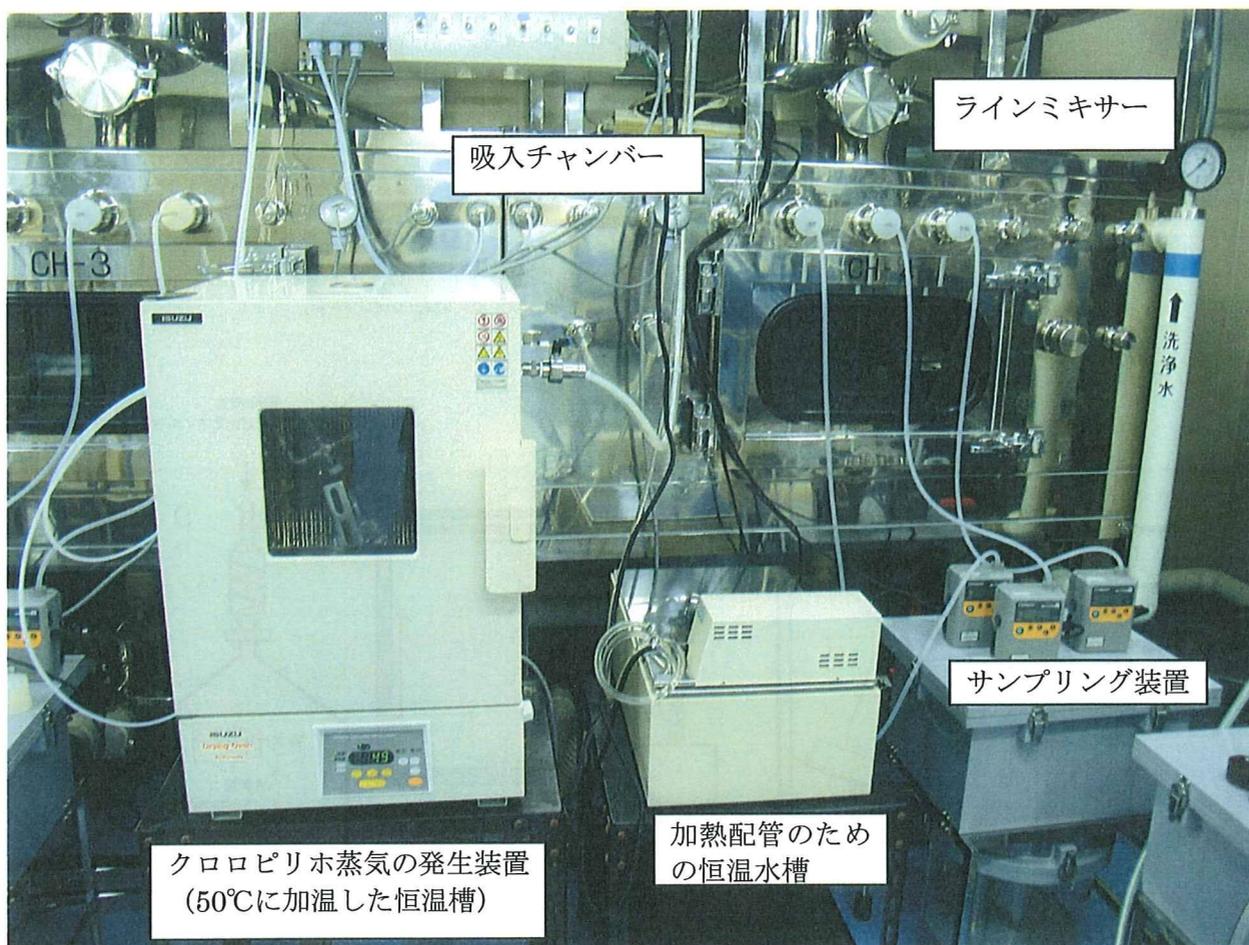|	設定条件1	設定条件2	設定条件3	設定条件4	設定条件5
暴露時間	6時間	6時間	6時間	22時間	22時間
発生容器を入れた恒温槽の温度	50℃	50℃	50℃	50℃	50℃
加熱配管の温度	50℃	50℃	50℃	50℃	50℃
希釈空気流量	212 L/分	212 L/分	212 L/分	212 L/分	212 L/分
目標濃度　0.07 ppb					
発生空気の流量	0.60 L/分	0.16 L/分	0.22 L/分	0.28 L/分	0.22 L/分
キャリア空気の流量	0.30 L/分	0.08 L/分	0.11 L/分	0.14L/分	0.11 L/分
測定値ppb（目標濃度に対する%）	0.308±0.007 (441%)	0.066±0.027 (94%)	0.056±0.003 (80%)	0.175±0.011 (251%)	0.129±0.001 (184%)
目標濃度　0.21 ppb					
発生空気の流量	1.70 L/分	0.34 L/分	0.38 L/分	0.39 L/分	0.38 L/分
キャリア空気の流量	0.80 L/分	0.17 L/分	0.19 L/分	0.195 L/分	0.19 L/分
測定値ppb（目標濃度に対する%）	1.061±0.009 (505%)	0.189±0.007 (90%)	0.207±0.006 (99%)	0.246±0.014 (117%)	0.272±0.013 (129%)
目標濃度　0.7ppb					
発生空気の流量	6.00 L/分	1.20L/分	0.80 L/分	0.92 L/分	0.80 L/分
キャリア空気の流量	3.00 L/分	0.60 L/分	0.4 0L/分	0.46 L/分	0.40 L/分
測定値ppb（目標濃度に対する%）	4.068±0.116 (581%)	1.075±0.008 (154%)	0.611±0.023 (87%)	0.883±0.016 (126%)	0.751±0.006 (107%)

網掛け：　変更した設定条件

図1　吸入暴露装置のシステム（クロルピリフォス）

図2　クロルピリフォスの吸入暴露装置

ラインミキサー

吸入チャンバー

サンプリング装置

加熱配管のための恒温水槽

クロロピリホ蒸気の発生装置（50℃に加温した恒温槽）

CH-3

洗浄水

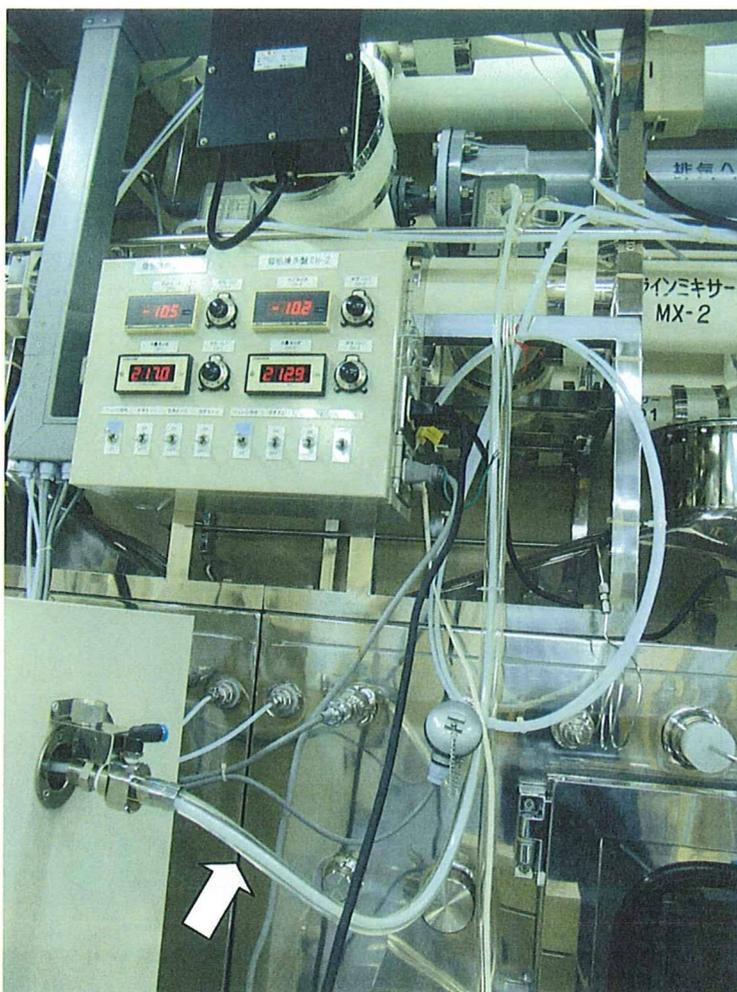図3　クロルピリフォスの発生容器（50℃に加温した恒温槽内に設置）

図4 クロルピリフォス蒸気をラインミキサーに送気するための
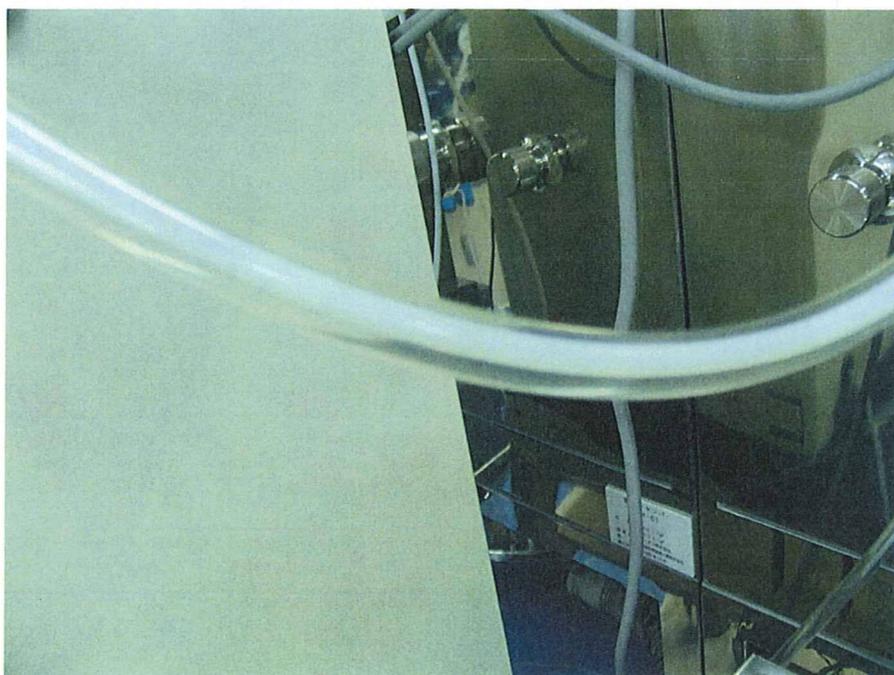　　加熱配管（50℃の温水で外周を加熱））

図5 図4の拡大（白色のテフロン管の外周に50℃の温水を循環し、加熱している）
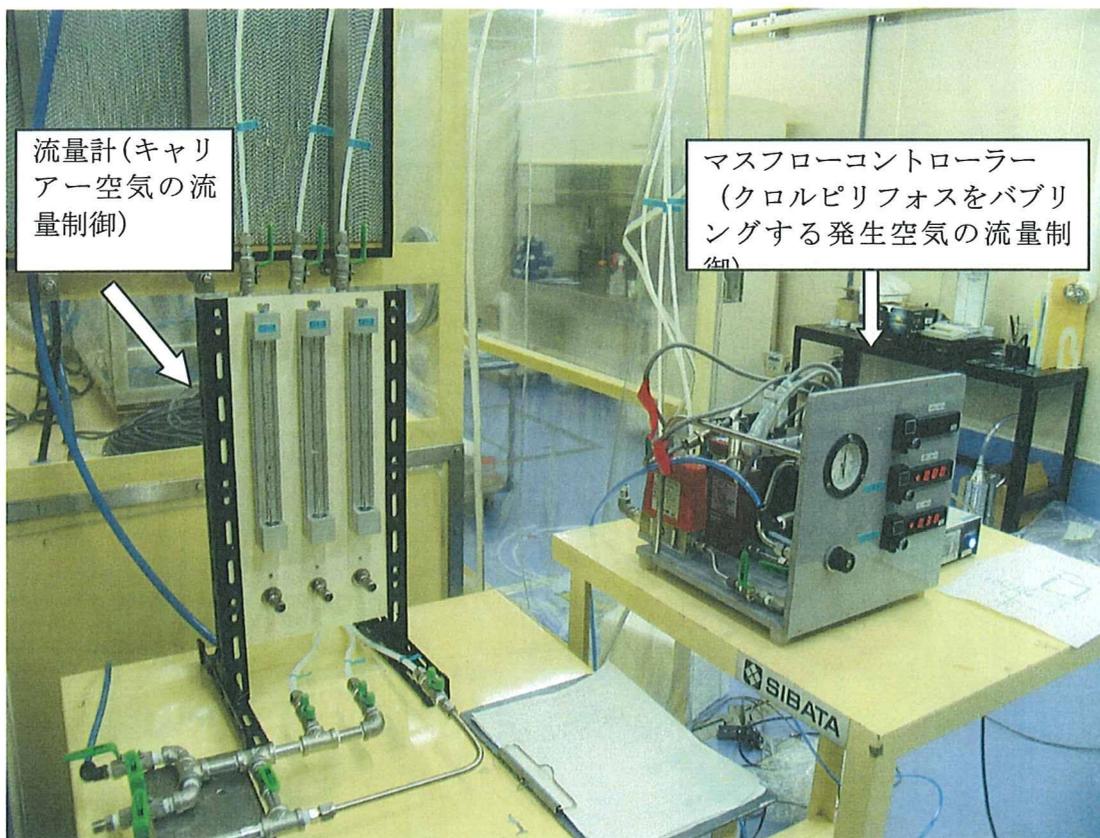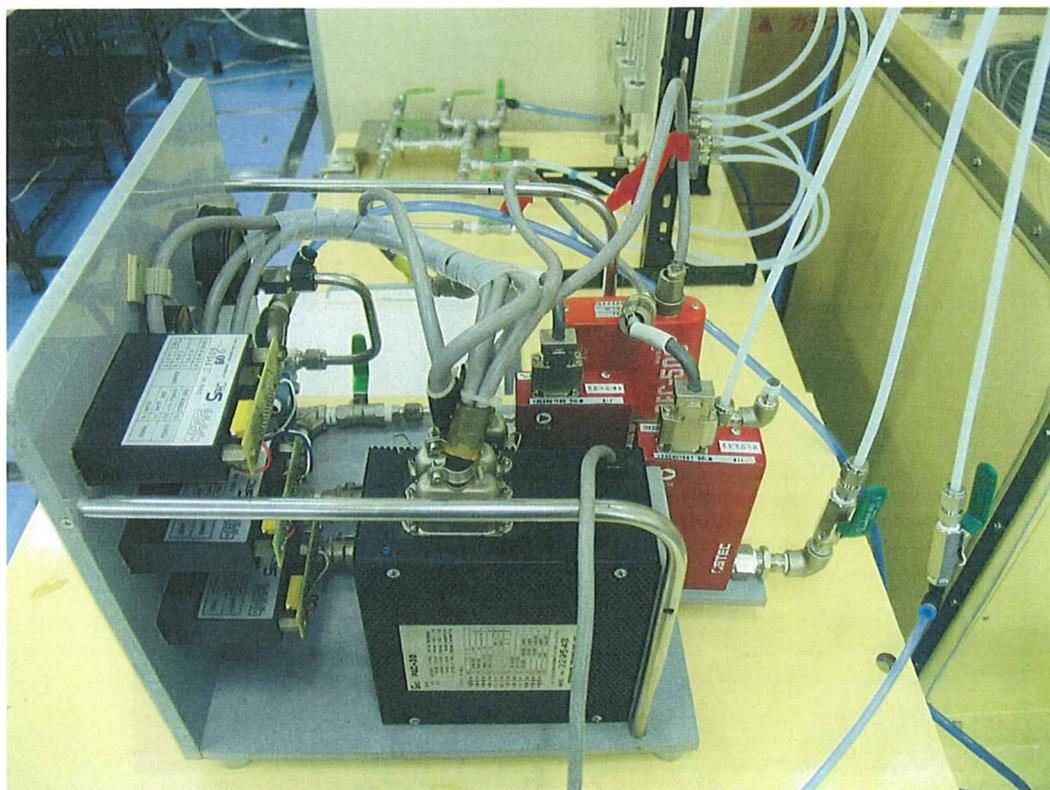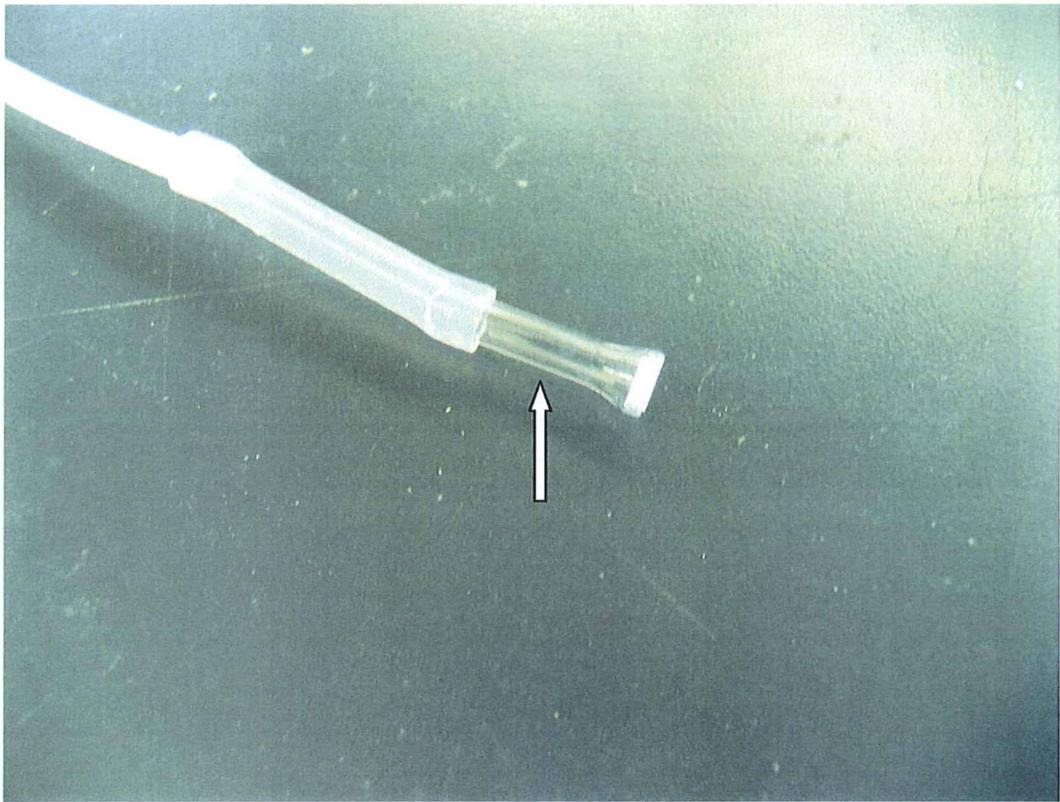
図6　クロルピリフォス蒸気の発生装置へ送る発生空気、キャリア空気の
　　　流量制御装置

図7　発生空気の流量を制御するマスフローコントローラー

図8　ゴアテックスチューブ（矢印）を装着したバブリング部分
　　　（先端部を閉じている）
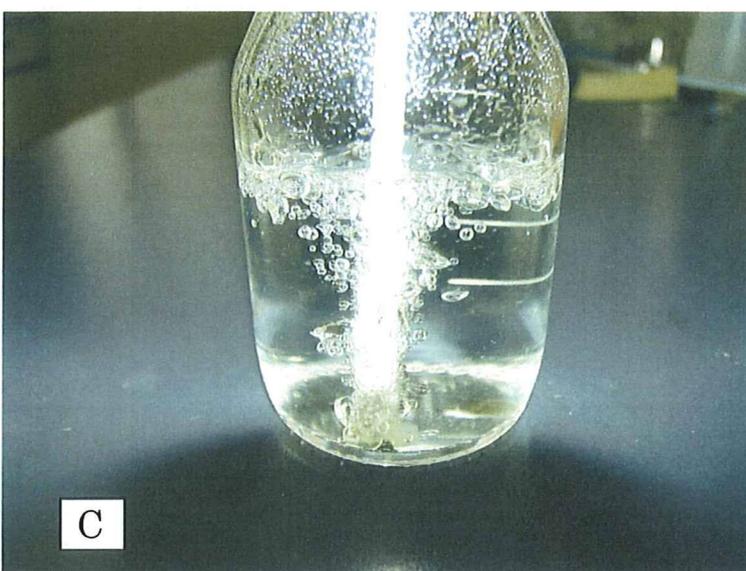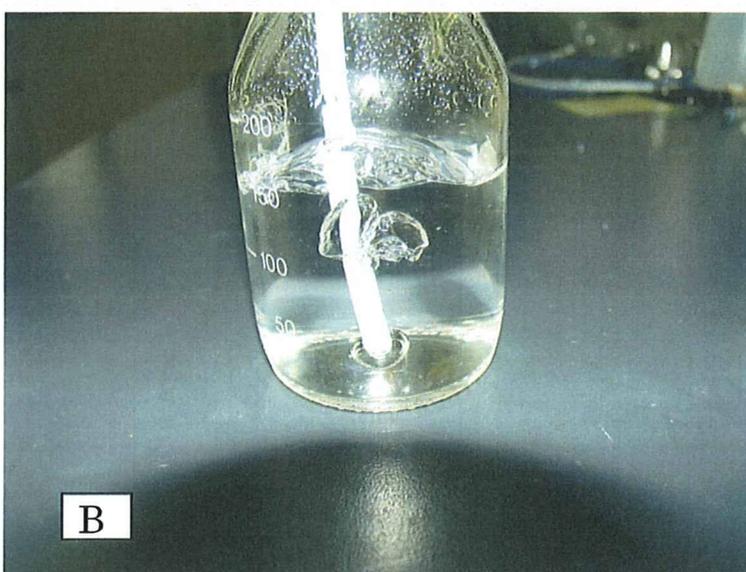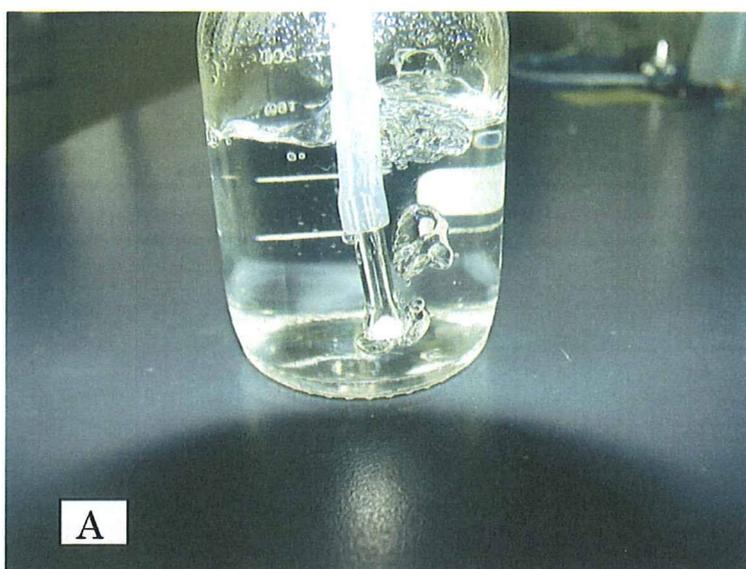
図9　バブリング部分の素材による気泡の発生状態の比較
　　A：ガラス管、B：テフロン管、C：先端を閉じたゴアテックスチューブ

別添 5

研究成果の刊行に関する一覧表

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Benzene-Induced Hematopoietic Neoplasms Including Myeloid Leukemia in *Trp*53-Deficient C57BL/6 and C3H/He Mice

Yasushi Kawasaki,*[,1] Yoko Hirabayashi,*[,2] Toyozo Kaneko,* Jun Kanno,* Yukio Kodama,* Yuuko Matsushima,* Yukio Ogawa,* Minoru Saitoh,* Kiyoshi Sekita,* Osayuki Uchida,* Takashi Umemura,* Byung-Il Yoon,*[,†] and Tohru Inoue[‡]

*Division of Cellular and Molecular Toxicology, Center for Biological Safety and Research, National Institute of Health Sciences, Tokyo 158-8501, Japan; †Laboratory of Histology and Molecular Pathogenesis, School of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Republic of Korea; and ‡Center for Biological Safety and Research, National Institute of Health Sciences, Tokyo 158-8501, Japan

This research focused on three major questions regarding benzene-induced hematopoietic neoplasms (HPNs). First, why are HPNs induced equivocally and at only threshold level with low-dose benzene exposure despite the significant genotoxicity of benzene even at low doses both in experiments and in epidemiology? Second, why is there no linear increase in incidence at high-dose exposure despite a lower acute toxicity ($LD_{50} > 1000$ mg/kg body weight; WHO, 2003, *Benzene in drinking-water. Background document for development of WHO Guidelines for Drinking-Water Quality*)? Third, why are particular acute myeloid leukemias (AMLs) not commonly observed in mice, although AMLs are frequently observed in human cases of occupational exposure to benzene? In this study, we hypothesized that the threshold-like equivocal induction of HPNs at low-dose benzene exposure is based on DNA repair potential in wild-type mice and that the limited increase in HPNs at a high-dose exposure is due to excessive apoptosis in wild-type mice. To determine whether *Trp*53 deficiency satisfies the above hypotheses by eliminating or reducing DNA repair and by allowing cells to escape apoptosis, we evaluated the incidence of benzene-induced HPNs in *Trp*53-deficient C57BL/6 mice with specific regard to AMLs. We also used C3H/He mice, AML prone, with *Trp*53 deficiency to explore whether a higher incidence of AMLs on benzene exposure might explain the above human-murine differences. As a result, heterozygous *Trp*53-deficient mice of both strains showed a nonthreshold response of the incidence of HPNs at the lower dose, whereas both strains showed an increasing HPN incidence up to 100% with increasing benzene exposure dose, including AMLs, that developed 38% of heterozygous *Trp*53-deficient C3H/He mice compared to only 9% of wild-type mice exposed to the high dose. The detection of AMLs in heterozygous *Trp*53-deficient mice, even in the C57BL/6 strain, implies that benzene may be a potent inducer of AMLs also in mice with some strain differences.

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[1] Deceased of apoplectic second cerebral attack on 6 August 2008 during follow-up study.

[2] To whom correspondence should be addressed at Division of Cellular and Molecular Toxicology, Center for Biological Safety and Research, National Institute of Health Sciences, 1-18-1 Kamiyohga, Setagayaku, Tokyo 158-8501, Japan. Fax: +81-3-3700-9647. E-mail: yokohira@nihs.go.jp.

The association between chronic benzene exposure and its effect of hematopoietic impairment was first observed in 1897 in tire workers by Santesson (1897). As additional cases were accumulated (Delore and Borgomano, 1928; Le Noir and Claude, 1897; Selling, 1910; Cabot, 1927; Smith, 1928), researchers found that benzene exposure induced not only bone marrow (BM) failure/aplastic anemia but also hematopoietic neoplasms (HPNs) including leukemias (Aksoy *et al.*, 1974; Penati and Vigliani, 1938). As reported in the literature, there is a narrow benzene exposure range for HPNs including leukemias and that for reversible or irreversible marrow aplasia both in humans and in experimental animals. The association between the benzene exposure and the cause of HPNs remained unclear until 1980, when Snyder *et al.* (1980) observed the first HPNs in mice induced by lifetime benzene exposure at 300 ppm, 6 h/day, and 5 days/week. Subsequently, Cronkite *et al.* (1982, 1984) confirmed the induction of HPNs through an exposure protocol that referenced the number of hematopoietic progenitor cells noted during the course of treatment.

The groundbreaking intermittent exposure protocols, developed by Cronkite *et al.* (1982, 1984), was originally intended not to exhaust the target cells but to maintain hematopoietic stem cells capable of transforming into HPNs. Indeed, a very high dose of benzene exposure in Swiss mice administered by gavage (500 mg/kg body weight for 4–5 days/week for 78 weeks) failed to induce any HPNs (Maltoni *et al.*, 1989), whereas exposure at lower doses of benzene for even 2 years by gavage using a protocol similar to that developed by the groups of Snyder and Cronkite (0, 25, 50, and 100 mg/kg body weight, 5 days/week) was found to induce HPNs at incidences of 8, 21, 20, and 31%, respectively (Huff *et al.*, 1989; NTP, 1986).

Subsequent studies further disclosed that benzene-induced hematotoxicity is mediated by aryl hydrocarbon receptors (Yoon *et al.*, 2002). The Snyder-Cronkite's protocol of

intermittent benzene inhalation was found to induce oscillatory proliferation of BM cells to counter any additional epigenetic hematopoietic neoplastic impacts (Yoon et al., 2001). DNA repair systems would naturally be affected by such epigenetic neoplastic impacts during intermittent oscillatory changes, and the weak oxidative stress induced by benzene metabolites has also been found to influence neoplastic transformation (Li et al., 2006; Snyder, 2007).

There remain some data gaps among the experimental animal studies of benzene-induced leukemias in this area. First, the incidence of HPNs after low-level benzene exposure in wild-type mice is threshold like and equivocal, despite the significant genotoxicity of benzene even at doses lower than 1 ppm and the related decrease in the number of hematopoietic progenitor cells (Lan et al., 2004). Second, there is a nonlinear-plateaued increase in the incidence of HPNs despite the lower toxicity of benzene (large LD_{50} value of 1000–10,000 mg/kg body weight; WHO, 2003). Third, there is a lack of acute myeloid leukemias (AMLs) in most of the experimental studies in mice, despite the high frequency of AMLs observed in human cases of occupational exposure to benzene.

Accordingly, reevaluation is required in order to resolve these data gaps. The equivocal response of the induction of HPNs at low doses is hypothesized on the basis of DNA repair mechanisms in wild-type mice, while a limited increase in the incidence of HPNs at high doses is hypothesized for a highly apoptosis-sensitive subfraction in the BM. In exploring this hypothesis, Trp53 deficiency may prove useful since this deficiency provides a cellular mechanism for the failure of DNA repair and for escape from apoptosis (French et al., 2001; Hirabayashi et al., 2003; MacDonald et al., 2004; Storer et al., 2001). Trp53-deficient C57BL/6 mice were used to evaluate the incidence of benzene-induced HPNs, specifically, in AMLs where there is a lack of DNA repair. Any potential increase in incidence of HPNs due to known Trp deficiency mechanisms may be interpreted in relation to murine AMLs. We can then compare the development of AML in these Trp53-deficient C57BL/6 mice to that seen in a C3H/He (AML prone) strain. This will make it feasible to identify any differences among strains regarding potentially excessive induction of leukemia associated with Trp53 deficiency.

Trp53-deficient mice show increased genomic instability and deficient repair mechanism because of the absence of cell cycle arrest induced by Trp53 after genotoxic damage. These mice, thus, provide a useful tool for examining an exaggerated neoplastic transformation after DNA damage induced by genotoxic chemicals. A marked increase in the incidence of chemical-induced cancers is potentially attributable to a genotoxic mechanism (Harvey et al., 1993; Hirabayashi et al., 2003; MacDonald et al., 2004; Kemp et al., 1994; Yoshida et al., 2007). This method has also been recommended as a sensitive experimental tool for carcinogenicity bioassay of directly genotoxic carcinogens, for ionizing radiation as well as for chemicals (French et al., 2001; Hirabayashi et al., 2003;

MacDonald et al., 2004; Storer et al., 2001). However, homozygous Trp53-deficient mice are difficult to utilize because of the high frequency of spontaneous thymic lymphomagenesis (Hirabayashi et al., 2003; MacDonald et al., 2004) due to the lack of physiological apoptosis in the double-negative immature T-cell subpopulation during the developmental stage (Haines et al., 2006).

Because the C3H/He strain exhibits a relatively high incidence of AML (Seki et al., 1991; Yoshida et al., 1996), the use of Trp53-deficient mice from both the C57BL/6 strain and the C3H/He strain may elucidate potential relationships and differences between benzene exposure and the development of AMLs in these two strains.

Owing to the high neoplastic sensitivity and myeloid leukemogenicity of the heterozygous Trp53-deficient C3H/He mice use in this study, exposure to benzene induced strain-dependent HPNs, including AMLs, in a nearly benzene dose–dependent manner, suggesting that our findings on the heterozygous Trp53-deficient mouse may provide a useful experimental model for studying benzene-induced hematotoxicity.

MATERIALS AND METHODS

Benzene. Benzene (CAS. no. 71-43-2, MW 78.11), widely utilized as a solvent for a various organic chemicals and present in gasoline and tobacco cigarettes, was obtained from Wako Fine Chemicals (Tokyo, Japan).

Animals. The targeting vector for Trp53, a recombinant with a 2.8-kb vector containing a neomycin-resistant gene immediately before the transcriptional start site, was inserted into TT2 embryonic stem cells (heterozygous for C57BL/6 and CBA; Yagi et al., 1993) to established homologous recombinant clones (Tsukada et al., 1993). By generating aggregation chimeras with this recombinant clones, chimeric mice and then Trp53-knockout mice were established in 1987 after confirmation of the germinal transmission in Trp53-deficient (C57BL/6 × CBA) F1 mice (Tsukada et al., 1993). General information on these recombinant mice is also found elsewhere (Trp53[tm1Sia] MGI: 1926340, Mouse Genome Informatics, 2009). The original Trp53-deficient (C57BL/6 × CBA) F1 mice backcrossed with C57BL/6 were transferred to the animal facility of the National Institute of Health Sciences (NIHS), Japan, in the second generation. Since then, the backcrossing with C57BL/6CrSlc was carried out for over 20 generations in 1997, followed by backcrossing with C3H/HeMsNrs in 2002. Both Trp53-deficient strains, C57BL/6 and C3H/He, were maintained by repeated backcrossing for each strain continuously.

This study used wild type, and homozygous and heterozygous Trp53-deficient male C57BL/6 and C3H/He mice were used. The heterozygous and homozygous Trp53-deficient mice and wild-type mice were generated by mating between heterozygous Trp53-deficient mice at the animal facility of NIHS, Japan. Neonates were genotyped using the primer for the targeted DNA sequence, including a partial neo gene at the 5' end partial exon 4, by PCR analysis using tissues obtained from the tail (Hirabayashi et al., 2002; Tsukada et al., 1993; Yoshida et al., 2002).

Cohort studies using 8-week-old mice were conducted using 10 mice for each genotype each time. Only male mice were studied in each strain owing to the similar incidences of HPN induction in both genders and to a limited number of rooms in the animal facility with gas chromatographs for the accurate monitoring of benzene exposure concentration. C57BL/6 mice (all genotypes) totaled 76 wild-type mice, 102 heterozygous Trp53-deficient mice, and 86 homozygous Trp53-deficient mice. All the animals were randomly selected on the basis of body weight and grouped by benzene dosage (300, 100,

TABLE 1

Incidences of Hematopoietic and Nonhematopoietic Diseases (histopathological types, C57BL/6 mice)

Genotype	Wild type				Heterozygous Trp53 deficiency				Homozygous Trp53 deficiency			
Benzene dose	0	33	100	300	0	33	100	300	0	33	100	300
No. of mice/group	20	19	19	18	24	27	25	26	21	19	23	23
HPNs (%)	2 (10.0)	4 (21.0)	3 (15.8)	10 (55.6)*	9 37.5%	11(40.7)	9 (36.0)	23 (88.5)*	19 (90.5)	18 (94.7)	22 (95.7)	17 (73.9)
Thymic lymphoma (%)	0 (0.0)	0 (0.0)	2 (10.5)	5 (27.8)*	0 (0.0)	1 (3.7)	4 (16.0)	19 (73.1)*	11 (52.4)	11 (57.9)	13 (56.5)	12 (52.2)
Nonthymic lymphoma (%)	2 (10.0)	4 (21.0)	1 (5.2)	5 (27.8)	9 (37.5)[a]	10 (37.0)	5 (20.0)	2 (7.7)*	8 (38.1)	7 (36.8)	8 (34.8)	5 (21.7)
Myeloid leukemia (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (7.7)	0 (0.0)	0 (0.0)	1 (4.3)	0 (0.0)
Other hematopoietic disorders (%)	2 (10.0)	1 (5.2)	1 (5.2)	0 (0.0)	5 (20.8)	4 (14.8)	1 (4.0)	1 (3.8)	2 (9.5)	0 (0.0)	0 (0.0)	0 (0.0)
Malignant fibrous histiocytoma (%)	0 (0.0)	1 (5.2)	1 (5.2)	0 (0.0)	5 (20.8)	3 (11.1)	0* (0.0)	0 (0.0)*	2 (9.5)	0 (0.0)	0 (0.0)	0 (0.0)
Myeloproliferative disorders/ myelodysplastic syndrome (%)	2 (10.0)	4 (21.1)[b]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Aplastic anemia/marrow failure (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.7)	1 (4.0)	1 (3.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Nonhematopoietic solid tumors (%)	3 (15.0)	3 (15.8)[b]	8 (42.1)	2 (11.1)	6 (25.0)[a]	12 (44.4)	8 (32.0)	2 (7.7)	0 (0.0)	1 (5.3)	1 (4.3)	6 (26.1)*
Non-neoplastic fatal diseases (%)	13 (65.0)	11 (57.9)[b]	7 (36.8)	6 (33.3)	5 (20.8)	0 (0.0)	7 (28.0)	0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a]Mouse that has two diseases.

[b]Other hematopoietic disorders and nonhematopoietic solid tumors or non-neoplastic fatal diseases overlapped.

*p Values < 0.05 between each sham control and treated group by Fisher's exact test.

33, and 0 ppm [sham exposure control]). Table 1 shows final numbers for all mice after the start of benzene exposure.

Totals for C3H/He mice were 70 wild-type mice, 72 heterozygous Trp53-deficient mice, and 60 homozygous Trp53-deficient mice. After random selection based on body weight, the mice were divided into three groups by benzene dosage (300, 100, and 0 ppm [as sham exposure control]). Table 2 shows final numbers for all mice after the start of benzene exposure.

During the study, the mice were housed individually within stainless wire cages, placed in inhalation chambers, and were kept on a 12-h light-dark cycle. An autoclave-sterilized basal pellet diet (CRF-1, Oriental Yeast Co., Ltd, Tokyo, Japan) was provided *ad libitum*, except during the 6-h daily inhalation time, when food was withdrawn irrespective of benzene treatment. Ultraviolet-sterilized water was supplied automatically via a tube throughout the study.

All the animals were maintained in a board-approved laboratory animal facility at NIHS, Japan. All experimental protocols involving the laboratory

mice used in this study were reviewed by the Interdisciplinary Monitoring Committee for Proper Animal Use and Welfare of Experimental Animals, a peer review panel established at NIHS, and approved by the Committee for Animal Care and Use (CACU) of the NIHS with the experimental code #473-2006. All animal studies were conducted using humane protocols approved by the CACU of the NIHS, Japan.

Benzene exposure. The mice were divided into the sham exposure control and benzene-exposed groups and housed in 1.3-m^3 horizontal lamina flow inhalation chambers with a flow rate of 650 l/min and 26 ventilation times/h (Sibata Scientific Technology Ltd., Tokyo, Japan) (Li et al., 2006; Yoon et al., 2001, 2002, 2003). The experimental groups were exposed to benzene at 300, 100, and 33 ppm for C57BL/6 mice and at 300 and 100 ppm for C3H/He mice, 6 h/day, 5 days/week for 26 weeks. The sham exposure control mice were maintained under the same conditions without benzene inhalation. After 26

TABLE 2

Incidences of Hematopoietic and Nonhematopoietic Diseases (histopathological types, C3H/He mice)

Genotype	Wild type			Heterozygous Trp53 deficiency			Homozygous Trp53 deficiency		
Benzene dose	0	100	300	0	100	300	0	100	300
No. of mice/group	23	24	23	24	24	24	18	20	22
HPNs (%)	2 (8.7)	6 (25.0)	7 (30.4)	6 (25.0)	20 (83.3)*	25 (104.2)*	15 (83.3)	14 (70.0)	20 (90.9)
Thymic lymphoma (%)	0 (0.0)	4 (16.7)	0 (0.0)	1 (4.2)	12 (50.0)*	6 (25.0)*[a]	12 (66.7)	12 (60.0)[a]	15 (68.2)[a]
Nonthymic lymphoma (%)	2 (8.7)[a]	2 (8.3)	5 (21.7)[a]	3 (12.5)	6 (25.0)	10 (41.7)*[a]	2 (11.1)	1 (5.0)	4 (18.2)
Myeloid leukemia (%)	0 (0.0)	0 (0.0)	2 (8.7)	2 (8.3)	2 (8.3)	9 (37.5)*	1 (5.6)	1 (5.0)	1 (4.6)[a]
Other hematopoietic disorders (%)	1 (4.4)	6 (25.0)	2 (8.7)	0 (0.0)	1 (4.2)	0 (0.0)	0 (0.0)	4 (20.0)	2 (9.1)
Malignant fibrous histiocytoma (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.2)	0 (0.0)	0 (0.0)	1 (5.0)	2 (9.1)
Myeloproliferative disorders/ myelodysplastic syndrome (%)	1 (4.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Aplastic anemia/marrow failure (%)	0 (0.0)	6 (25.0)*	2 (8.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (15.0)	0 (0.0)
Nonhematopoietic solid tumors (%)	11 (47.8)[a]	5 (20.8)*	8 (34.8)[a]	11 (45.8)	2 (8.3)*	0 (0.0)*	0 (0.0)	2 (10.0)[a]	1 (4.6)
Non-neoplastic fatal diseases (%)	10 (43.5)	7 (29.2)	7 (30.4)	7 (29.2)	1 (4.2)*	0 (0.0)*	3 (16.7)	1 (5.0)	0 (0.0)

*p Values < 0.05 between each sham control and treated group by Fisher's exact test.

[a]Mouse that has two diseases.

weeks, all the animals were observed throughout their lifetime under the same conditions without benzene inhalation.

Dose monitoring for benzene exposure. The benzene atmosphere was generated by heating liquid benzene to 16°C to form a vapor (Sibata Scientific Technology Ltd). A gas chromatograph (Shimadzu Co., Kyoto, Japan) was used to measure benzene concentration in the chambers at 30-min intervals during daily exposures (Shimadzu Co.) (Li *et al.*, 2006; Yoon *et al.*, 2001, 2002, 2003). The temperature and humidity in the chambers were automatically controlled at 24°C ± 1°C and 55 ± 10%, respectively.

PCR analysis for genotyping. To detect *Trp*53 wild-type and *Trp*53-deficient alleles, PCR analysis was performed using genomic DNA extracted from the tail of each mouse, and synthetic oligonucleotides were used as primers as described elsewhere (Tsukada *et al.*, 1993) and briefly here as follows. To detect the *Trp*53 wild-type allele, the common 5' primer (5'-aattgacaagttatgcatcca-3') and 3' primer (5'-actcctcaacatcctggggcagcaaca-gat-3') were used. To detect the *Trp*53-deficient allele, the common 5' primer and *neo sequence* primer (5'-gaacctgcgtgcaatccatcttgttcaatg-3') were used.

Lifetime observation. All mice were monitored at least twice daily throughout their lifetime. Those showing fatal symptoms, including advanced leukemias, such as anemia with pale extremities and palpable splenomegaly, were euthanized at the agonal period and then examined hematopathologically and histopathologically. Mice that died were examined for their gross anatomical features, after which all visceral organs were fixed in 10% neutral buffered formalin for histopathological examination.

Histopathological examination. All visceral organs, including the thymus, spleen, sternum, and femoral BM, were fixed in 10% neutral buffered formalin for 24 h. The sternum and femoral BM were decalcified in 7.5% formic acid for 72 h. After conventional processing for dehydration, paraffin-embedded sections were stained with hematoxylin and eosin and then examined histopathologically under a light microscope (Frith *et al.*, 2001; Hirabayashi *et al.*, 1992).

Loss of heterozygosity. During the course of benzene-induced leukemo-genesis, the remaining wild-type allele of *Trp*53 remaining in heterozygous *Trp*53-deficient mice may be inactivated. The frequency of such loss of heterozygosity (LOH) was previously evaluated in mice with radiation-induced leukemias. LOH for the remaining *Trp*53 allele was not examined in each group because high level of consistency (91.7%) had been identified in the leukemogenicity assay previously conducted for this strain at our laboratory (Yoshida *et al.*, 2007).

Statistical analyses. Survival curves data were stored in a computer and processed for statistical analysis to obtain mean survival time and SE by the Kaplan-Meier method and to evaluate statistical significance by the log-rank test using SPSS 14.1 (SPSS, Inc., Chicago, IL). To determine the cumulative incidences of diseases, Fisher's exact test was applied using Microsoft Office Excel 2003 (Microsoft, Redmond, WA). Differences were considered significant at $p < 0.05$.

RESULTS

Survival Curves with Graded Exposure Doses of Benzene Inhalation

Experimental groups. Kaplan-Meier survival curves for wild-type mice in comparison to the two strains (C57BL/6 and C3H/He) of heterozygous and homozygous *Trp*53-deficient mice are shown in Figures 1A–1C and Figures 1D–1F, respectively. Figures 1A–C show data for C57BL/6 mice of different genotypes, classified into four groups on the basis of benzene exposure (33, 100, and 300 ppm, 6 h/day, 5

days/week, for 26 weeks, and 0 ppm as the sham exposure control). Figures 1D–1F show data from C3H/He mice of different genotypes classified into three groups on the basis of benzene exposure dose (100 and 300 ppm, 6 h/day, 5 days/week, for 26 weeks, and 0 ppm as the sham exposure control).

C57BL/6 strain. The mean survival time for C57BL/6, wild-type mice in the sham exposure control group, was 629 ± 40 days (mean ± SE) after the start of the experiment (Fig. 1A). The mean survival time for the wild-type mice in the 33- and 100-ppm exposure groups was 635 ± 40 and 550 ± 41 days, respectively, and the mean survival times for wild-type mice in the 300-ppm exposure group was 346 ± 30 days (mean ± SE). Survival time decreased proportionally with increasing benzene exposure except for the slight overlapping of survival curves for the 33-ppm and the sham exposure control groups.

Among heterozygous *Trp*53-deficient mice in the 300-ppm exposure group (Fig. 1B), the survival curve shows a rapid decrease in the number of surviving mice. Mean survival time in this group was 163 ± 9 days (mean ± SE) after the start of exposure, in comparison to 346 ± 30 days in the wild-type mice. Thus, the mean survival times for the heterozygous *Trp*53-deficient group and the wild-type group, both exposed to 300 ppm, were 347 and 283 days, respectively, shorter than the corresponding sham exposure control groups (510 ± 25 days for the heterozygous *Trp*53-deficient group and 629 ± 40 days for the wild-type group).

C3H/He strain. In the C3H/He wild-type sham exposure group, survival time was 590 ± 33 days (mean ± SE) after the start of exposure (Fig. 1D). Mean survival time in the wild-type 100-ppm exposure group was 495 ± 39 days and in the wild-type 300-ppm exposure group was 353 ± 35 days (mean ± SE in both cases). In contrast, within the heterozygous *Trp*53-deficient group exposed to 300-ppm benzene by inhalation, the first death occurred about 71 days after the start of exposure, and mean survival time ± SE was 117 ± 5 days (Fig. 1E).

Homozygous Trp53-deficient mice. All mice in both strains with homozygous *Trp*53 deficiencies died relatively soon (Figs. 1C and 1F), with mean survival times ranging 16–122 days, regardless of benzene exposure including 0 ppm. All survival curves, specifically in the four C57BL/6 groups, crossed or nearly crossed each other, except for longer survival in a small number of mice (less than 5%) in the 100-ppm exposure group. We attribute this to primarily thymic lymphomas that originate in double-negative CD4/CD8 cells lacking apoptosis, so our findings in homozygous *Trp*53-deficient mice have been omitted from further discussion. In the C3H/He mice, however, Kaplan-Meier comparison showed a statistically significant difference in survival curves between the 300-ppm exposure group and the sham exposure as determined by the log-rank test ($p = 0.002$, data not shown).
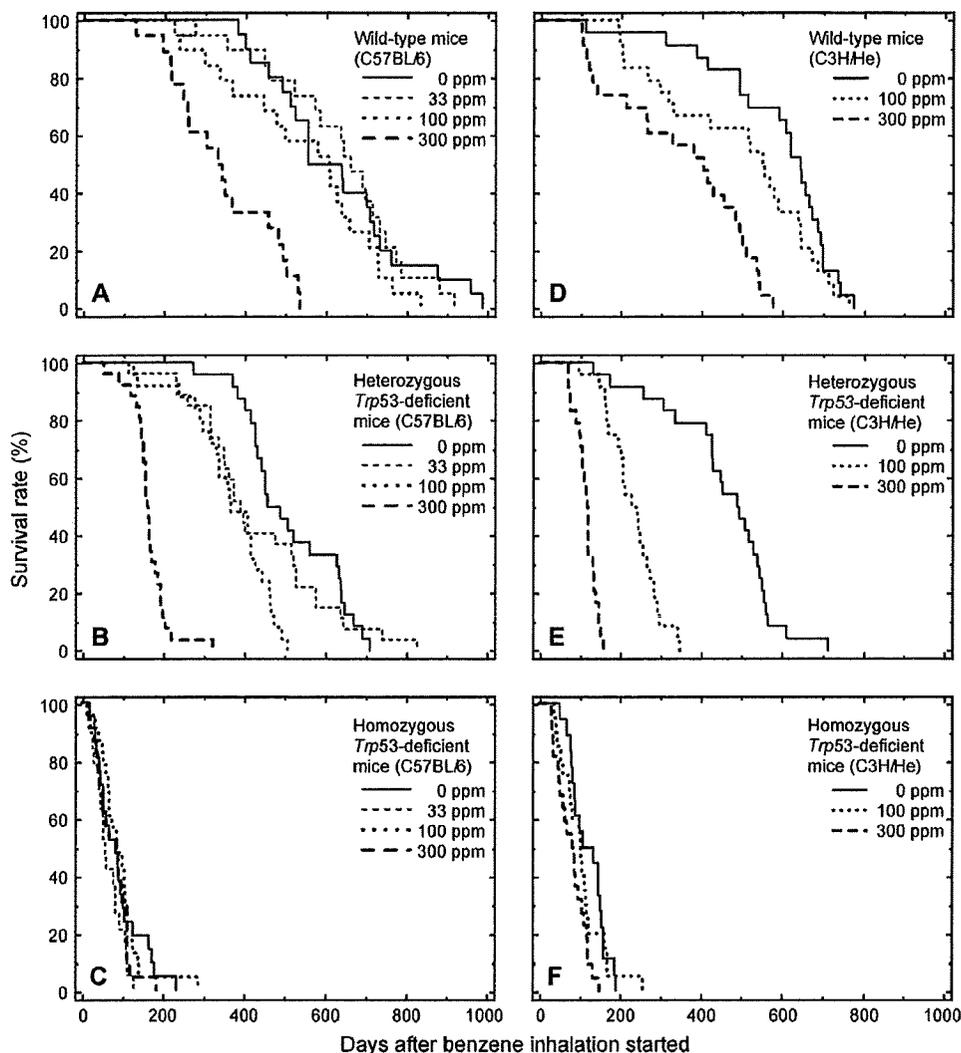
FIG. 1. Survival rates (%) are plotted on the vertical axis and survival time (days) after benzene inhalation on the horizontal axis, using (A)–(C) for C57BL/6 mice and (D)–(F) for C3H/He mice, with (A) and (D) for wild-type mice, (B) and (E) for heterozygous *Trp53*-deficient mice, and (C) and (F) for homozygous *Trp53*-deficient mice. Bold dotted lines for 300-ppm exposure group, regular dotted line for 100-ppm exposure group, fine dotted lines for 33-ppm exposure group, and solid line for sham exposure control. Statistical significance by log-rank test: (A) 0 versus 300 ppm, $p = 4.7 \times 10^{-06}$; 33 versus 300 ppm, $p = 8.3 \times 10^{-06}$; 100 versus 300 ppm, $p = 4.9 \times 10^{-04}$; (B) 0 versus 300 ppm, $p = 1.4 \times 10^{-10}$; 33 versus 300 ppm, $p = 4.8 \times 10^{-10}$; 100 versus 300 ppm, $p = 2.2 \times 10^{-08}$; 0 versus 100, $p = 1.9 \times 10^{-04}$; 33 versus 100 ppm, $p = 3.5 \times 10^{-02}$; (C) no significant difference between groups; (D) 0 versus 300 ppm, $p = 5.6 \times 10^{-06}$; 100 versus 300 ppm, $p = 1.0 \times 10^{-03}$; (E) 0 versus 300 ppm, $p = 5.8 \times 10^{-06}$; 100 versus 300 ppm, $p = 4.0 \times 10^{-09}$; 0 versus 100 ppm, $p = 1.1 \times 10^{-07}$; and (F) 0 versus 300 ppm, $p = 4.4 \times 10^{-03}$.

Cumulative Deaths due to HPNs

Benzene exposure in wild-type mice. The cumulative incidences of HPNs in each wild-type experimental group are shown in Figure 2A (C57BL/6) and Figure 2D (C3H/He). In C57BL/6 mice, the wild-type group exposed to 300 ppm showed a gradual increase in cumulative incidence of HPNs to 55.6% by day 532. In C3H/He mice, groups exposed to 100 and 300 ppm showed somewhat lower but similar increases in HPNs to 25.0% by 554 days and 30.4% by 431 days, respectively, as seen in Figure 2A (C57BL/6) and Figure 2D

(C3H/He). With the exception of the 300-ppm exposure group of wild-type C57BL/6 mice, the incidence and onset of HPNs did not exceed 21.0% during lifetime observation (21.0% for the 33-ppm group and 15.8% for the 100-ppm groups). The maximum incidences of HPNs in the wild-type sham control group were 10.0% by 492 days in C57BL/6 mice and 8.7% by 742 days in C3H/He mice.

The first question in the present study concerned threshold-like equivocal incidence of HPNs at low-dose benzene exposure. In this regard, only the C57BL/6, 300-ppm exposure
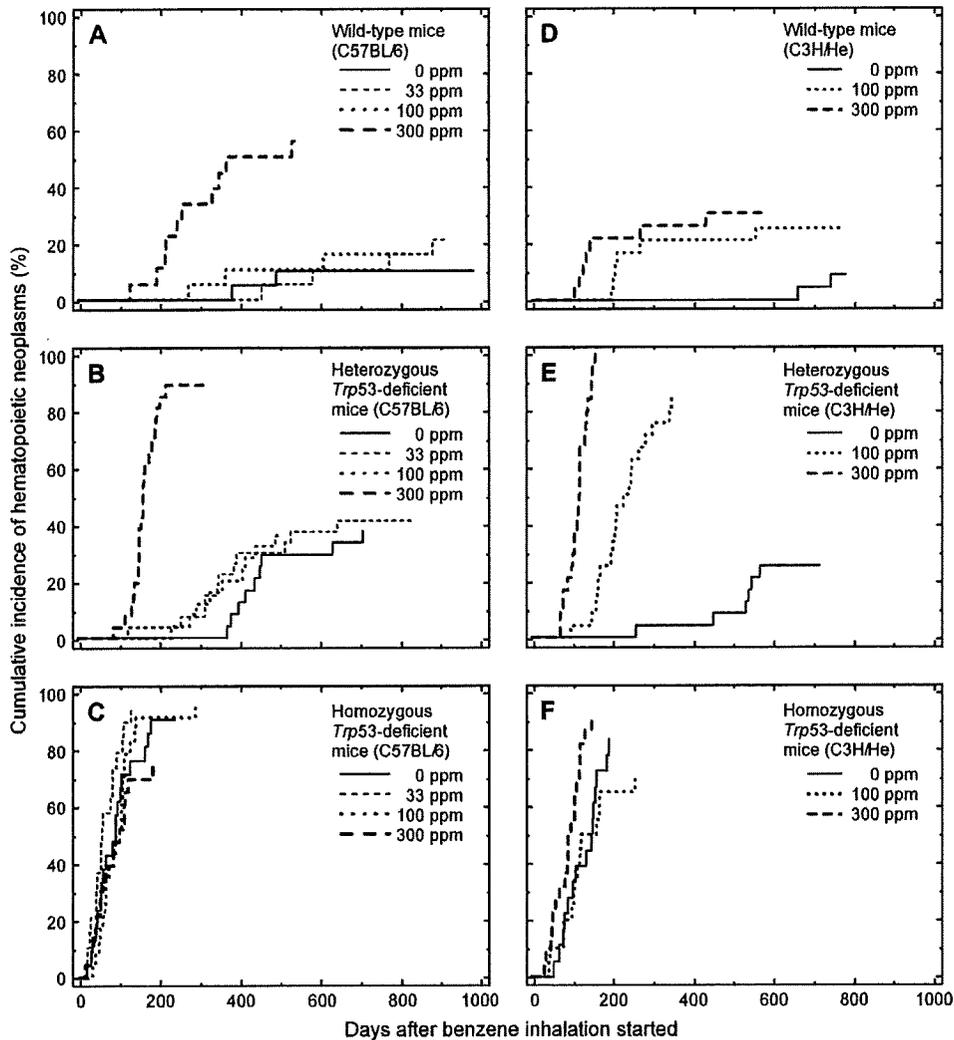
FIG. 2. The illustration shows the cumulative lifetime incidence of all hematopoietic malignancies (%) in C57BL/6 mice on the left (A–C) and in C3H/He mice on the right (D–F), using (A) and (D) for wild-type mice, (B) and (E) for heterozygous p53-deficient mice, and (C) and (F) for homozygous p53-deficient mice. Bold dotted line for 300-ppm exposure group, regular dotted line for 100-ppm exposure group, fine dotted line for 33-ppm exposure group, and solid line for sham exposure control. Statistical significance by log-rank test: (A) 0 versus 300 ppm, $p = 2.7 \times 10^{-04}$; 33 versus 300 ppm, $p = 4.5 \times 10^{-04}$; 100 versus 300 ppm, $p = 1.8 \times 10^{-03}$; (B) 0 versus 300 ppm, $p = 2.5 \times 10^{-10}$; 33 versus 300 ppm, $p = 8.6 \times 10^{-10}$; 100 versus 300 ppm, $p = 9.1 \times 10^{-10}$; (C) no significant difference between groups; (D) 0 versus 300 ppm, $p = 7.0 \times 10^{-03}$; (E) 0 versus 300 ppm, $p = 1.2 \times 10^{-11}$; 100 versus 300 ppm, $p = 2.8 \times 10^{-09}$; 0 versus 100 ppm, $p = 4.2 \times 10^{-08}$; and (F) 0 versus 300 ppm, $p = 3.1 \times 10^{-03}$; 100 versus 300 ppm, $p = 2.1 \times 10^{-02}$.

group showed a significant differences in cumulative HPN incidence in comparison to the other C57BL/6 groups. However, findings from both the C3H/He 300-ppm and the 100-ppm exposure groups differed significantly from the sham exposure controls. These results imply that HPNs occurred at a higher than threshold level in heterozygous Trp53-deficient mice in both strains since such incidence was greater than and clearly separated from the incidence in each sham exposure control groups.

Exposure in Trp53-deficient mice. A high frequency of HPNs was observed in both strains of the heterozygous Trp53-deficient benzene exposure groups as shown in Figure 2B

(C57BL/6) and Figure 2E (C3H/He). In heterozygous Trp53-deficient C57BL/6 mice, a total HPN incidence of 88.5% (300 ppm) was observed from 88 to 219 days. This incidence was higher than in the sham exposure control (37.5%) and also higher than in the wild-type groups with or without benzene exposure (55.6 and 10.0%, respectively) and with earlier onset time (88 days) than in wild-type mice (130 days). The increase in incidence of HPNs between benzene exposure group and sham control was not greater in Trp53-deficient C57BL/6 mice than in the wild-type mice due to an increase in the late-appearing spontaneous HPNs in the Trp53-deficient mice, but the 50% die-off time (days) for HPNs between the former and
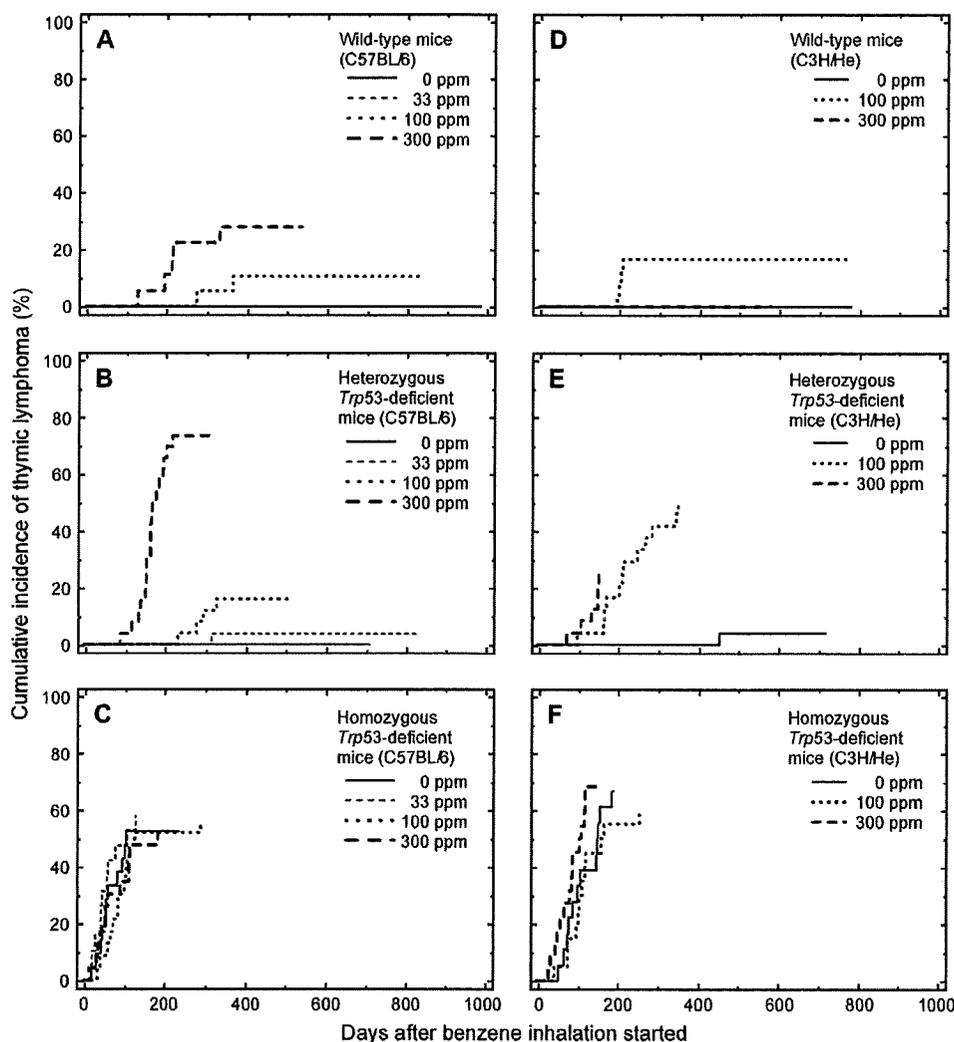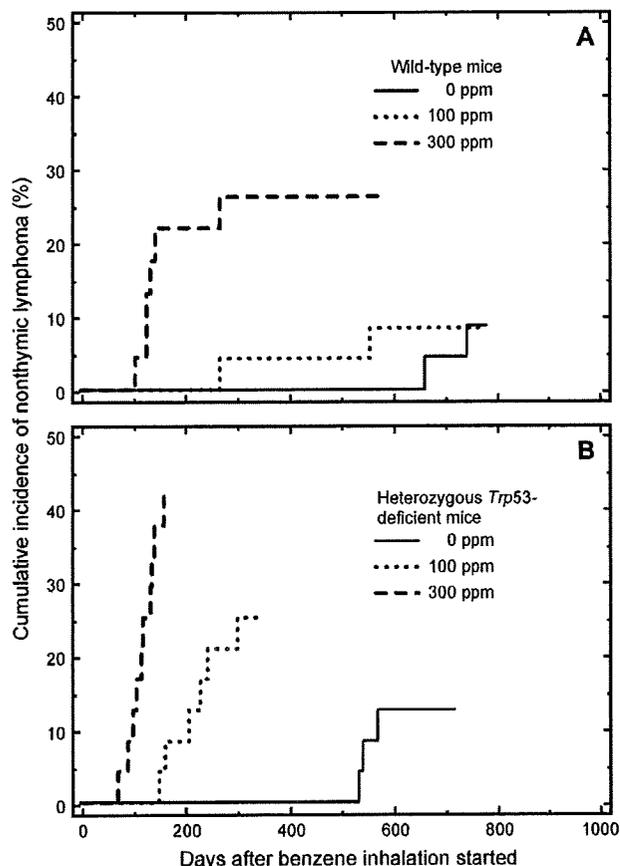
FIG. 3. Cumulative lifetime incidences of thymic lymphoma during the lifetime in C57BL/6 mice on the left (A–C) and C3H/He mice on the right (D–F), using (A) and (D) for the wild-type mice, (B) and (E) for heterozygous *Trp53*-deficient mice, and (C) and (F) for homozygous *Trp53*-deficient mice. Bold dotted line for 300-ppm exposure group, regular dotted line for 100-ppm exposure group, fine dotted line for 33-ppm exposure group, and solid line for sham exposure control. Statistical significance by log-rank test: (A) 0 versus 300 ppm, $p = 9.7 \times 10^{-03}$; 33 versus 300 ppm, $p = 1.2 \times 10^{-02}$; (B) 0 versus 300 ppm, $p = 4.3 \times 10^{-10}$; 33 versus 300 ppm, $p = 1.7 \times 10^{-10}$; 100 versus 300 ppm, $p = 6.6 \times 10^{-09}$; 0 versus 100 ppm, $p = 3.4 \times 10^{-02}$; (C) no significant difference between the two groups; (D) 0 versus 100 ppm, $p = 4.8 \times 10^{-02}$; (E) 0 versus 300 ppm, $p = 3.9 \times 10^{-05}$; 100 versus 300 ppm, $p = 2.4 \times 10^{-03}$; 0 versus 100 ppm, $p = 2.4 \times 10^{-06}$; and (F) 0 versus 300 ppm, $p = 3.0 \times 10^{-02}$.

the latter was significantly split in the *Trp53*-deficient mice than in the wild-type mice (266.5 vs. 184.5 days). The cumulative incidence curves for HPN in these heterozygous *Trp53*-deficient exposure groups were significantly different only in the 300-ppm exposure group, and the curves of the remaining groups occasionally overlapped for the C57BL/6 strain, but the benzene dose–dependent shortening of 50% die-out time in the 100-ppm group and the die-out time in the 100-ppm *Trp53*-deficient groups were both similarly reduced (70.5 and 70.0 days).

In heterozygous *Trp53*-deficient C3H/He mice, in contrast, the total incidence of HPNs increased in a manner dependent

on the benzene exposure dose (104.2, 83.3, and 25.0%, respectively), with earlier onset times (78, 98, and 260 days) than in wild-type mice (105, 197, and 651 days).

As illustrated in Figure 2C (C57BL/6) and Figure 2F (C3H/He), although homozygous *Trp53*-deficient mice showed slightly earlier onset of thymic lymphomas following benzene exposure, specifically in the C3H/He strain, these mice were not used for bioassay because they showed extremely early onset of highly frequent thymic lymphomas that developed spontaneously by a known mechanism, that is, development of CD4/CD8 double-negative thymic lymphomas owing to the
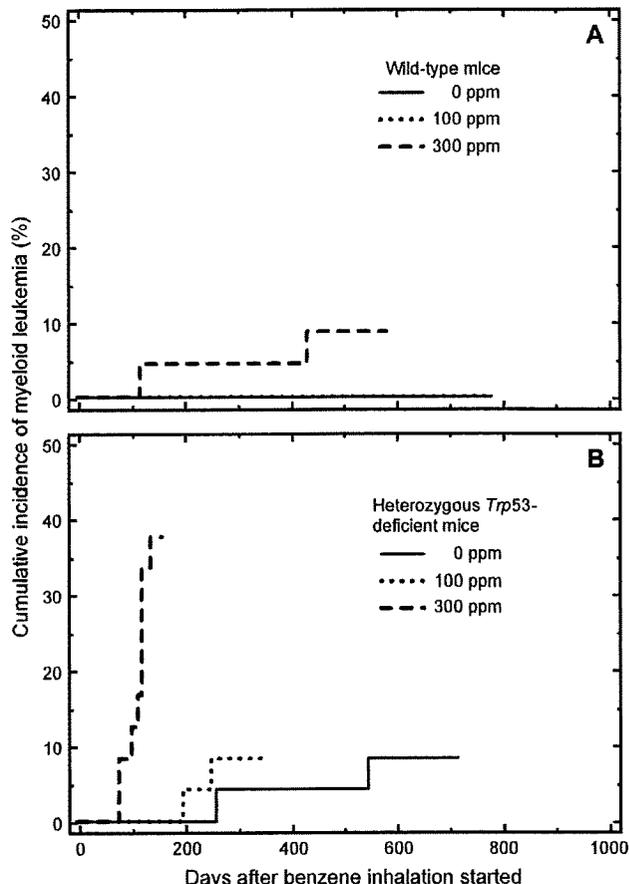
FIG. 4. Cumulative lifetime incidence of nonthymic (non-Hodgkin) lymphoma in C3H/He mice; wild-type mice (A) and heterozygous Trp53-deficient mice (B). Bold dotted line for 300 ppm, regular dotted line for 100 ppm, and solid line for sham exposure control. Statistical significance determined by log-rank test: (A) 0 versus 300 ppm, $p = 3.1 \times 10^{-02}$; and (B) 0 versus 300 ppm, $p = 1.4 \times 10^{-05}$; 100 versus 300 ppm, $p = 6.4 \times 10^{-05}$; 0 versus 100 ppm, $p = 4.0 \times 10^{-03}$.

FIG. 5. Cumulative lifetime incidences of myelogenous leukemia during the lifetime of C3H/He mice; wild-type mice (A) and heterozygous Trp53-deficient mice (B). Bold dotted lines for 300 ppm, regular dotted lines for 100 ppm, and solid lines for sham exposure controls. Statistical significance determined by log-rank test: (A) no significant difference between groups; (B) 0 versus 300 ppm, $p = 1.5 \times 10^{-04}$; 100 versus 300 ppm, $p = 1.8 \times 10^{-04}$.

absence of physiological apoptosis in the CD4/CD8 double-negative immature T-cell population during early development (Haines et al., 2006).

Histopathological Examination

HPNs, along with non-HPNs and non-neoplastic diseases observed in C57BL/6 mice and C3H/He mice, were classified histopathologically and tabulated separately in Table 1 for the C57BL/6 strain and Table 2 for the C3H/He strain.

Development of thymic and nonthymic lymphoma. As shown in these tables, in wild-type mice, only a small number of HPNs, that is, thymic lymphomas, two (10.5%) and five (27.8%) in the C57BL/6 and four (16.7%) and zero (0%) in the C3H/He, were observed in 100- and 300-ppm exposure groups, respectively (Figs. 3A and 3D). In heterozygous Trp53-deficient C57BL/6 mice, the number of thymic lymphomas gradually increased, that is, 0, 1 (3.7%),

4 (16.0%), and 19 (73.1%), with benzene exposure dose, that is, 0, 33, 100, and 300 ppm, respectively (Fig. 3B). Thus, the graded increase in the incidence of thymic lymphomas up to 73.1% was observed in the C57BL/6 strain, showing a linear exposure dose-response relationship. In C3H/He mice, on the other hand, the number of thymic lymphomas that developed were 1 (4.2%), 12 (50.0%), and 6 (25.0%) at benzene exposure doses of 0, 100, and 300 ppm, respectively (Fig. 3E). Thus, the number of thymic lymphomas at 300 ppm decreased and a linear exposure dose-response relationship was not observed. The mechanism underlying this observation needs to be studied.

Concerning the incidence of nonthymic (non-Hodgkin) lymphomas, a linear exposure dose-response relationship was not observed in the C57BL/6 strain, but relative increases in the incidence with the exposure dose of benzene were observed in C3H/He mice (Figs. 4A and 4B).
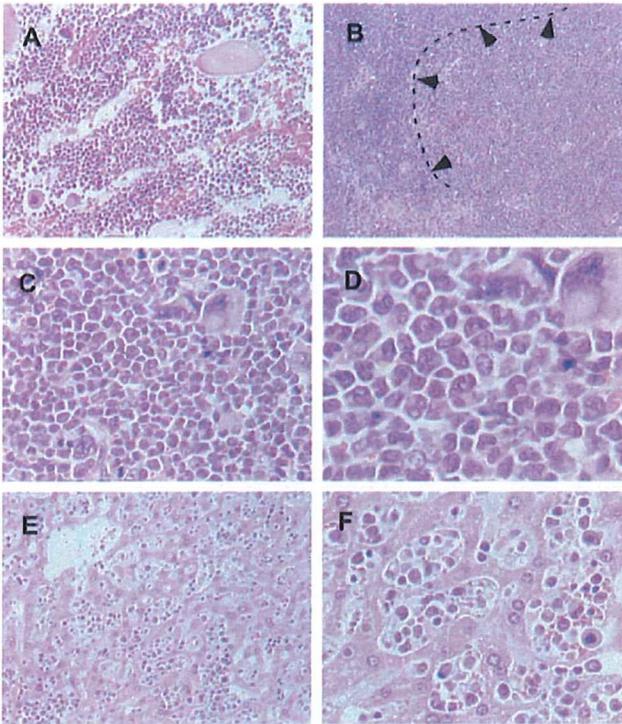
FIG. 6. Representative histopathological findings for AMLs developing in benzene-exposed wild-type C3H/He mice (A–F). Increased cellular density for atypical mononuclear cells with heterogeneous size distribution in sternum BM (A, ×67). Increased cellular density with expanding growth (arrow heads) toward surrounding splenic tissue and the lymphofollicular structures on the left (B, ×34). Higher magnification of neoplastic cellular component at the center of leukemic growth in (B), consisting of atypical myeloid cells with scattered bizarrely shaped myeloblastic nuclei including megakaryocytes (C, ×169). Higher magnification of (C), revealing detailed atypical myeloid cells, including cells with occasional doughnut-shaped nuclei (D, ×312). Hepatic cell cord filled with atypical mononuclear myeloblastic cell component surrounding a central vein at the upper left (E, ×67). Higher magnification of (E), including atypical myeloid cells, with heterogeneous size distribution, proliferating in sinusoidal spaces (F, ×169).

Development of AMLs. It is notable that heterozygous *Trp*53-deficient C3H/He mice, which are prone to AML, produced two (8.3%), two (8.3%), and nine (37.5%) AMLs in the 0-, 100-, and 300-ppm exposure groups, respectively, in comparison with wild-type mice, which produced only two (8.7%) AMLs in the 300-ppm exposure group (Fig. 5). In C57BL/6 mice, there were two AMLs in heterozygous and one in homozygous *Trp*53-deficient animals. There were essentially no significant differences in cytological and histopathological findings of AMLs between the both strains. Thus, mainly cytological and histopathological findings of AMLs developed in C3H/He mice are shown in Figure 6 (leukemias developing in wild-type mice) and Figure 7 (leukemias developing in *Trp*53-deficient mice), along with two panels (7E and 7F) from heterozygous *Trp*53-deficient C57BL/6 mice in Figure 7, bottom.

In Figure 6, atypical myeloblastic leukemic cells with irregularly bizarrely shaped nuclei, occasionally including

doughnut-shaped nuclei as shown in Figures 6C and 6D, suggest a myelogenous origin in C3H/He mice. The same atypical myeloid cells with a heterogeneous size distribution were observed to invade hepatic sinusoidal spaces (Figs. 6E and 6F). In wild-type mice, AMLs developed only in the C3H/He mice and not in the C57BL/6 mice.

Owing to the function of *Trp*53 during the early developmental stage, a prominently lesser extent of differentiation was noted in AMLs developing in *Trp*53-deficient mice. Namely, as shown in Figure 7, the cytopathological and histopathological characteristics of leukemic cells in both heterozygous *Trp*53-deficient C3H/He mice (Figs. 7A–D) and C57BL/6 mice (Fig. 7E) revealed more immature blastic cells with less differentiation than leukemic cells in wild-type mice (Fig. 6). Representative atypical myeloblastic cells possessing trace peroxidase granules in the cytoplasm are shown in Figure 7B (inset, bottom). Nevertheless, some doughnut-shaped nuclei similar to those of cells with myeloid lineages were very occasionally observed in the C57BL/6 strain (Fig. 7E, inset, top and bottom).

HPNs in Relationship to Benzene Exposure Dose

The exposure dose range for benzene hematotoxicity is narrow, specifically for the induction of HPNs. Higher benzene exposure may produce a larger number of hematopoietic neoplastic candidates but simultaneously seems to decrease the number of hematopoietic progenitor cells, that is, potential targets for the induction of HPNs. Figures 8A and 8B (for C57BL/6 mice) and Figures 8C and 8D (for C3H/He mice) illustrate the relationship between the incidence of HPNs and graded increased benzene exposure.

In C57BL/6 mice, the increase in the total incidence of HPNs was only significant in both the 300-ppm exposure groups for wild-type and the heterozygous *Trp*53-deficient mice. Each histological type showed a statistically significant increase in the incidence of thymic lymphoma at 300-ppm exposure in comparison to sham exposure (Table 1). There was no statistically significant increase in HPN incidence in either the 33- or 100-ppm exposure group in comparison to spontaneous HPNs in the sham exposure groups, possibly due to the competitive increase in the incidence of non-HPNs.

In the C3H/He mice, however, the total incidence curve for HPNs in wild-type mice showed a gradual increase reaching a plateau/peak in the wild-type 100- and 300-ppm exposure groups (Fig. 8C). The heterozygous *Trp*53-deficient mice showed a significant increase (*) in HPN incidence in the 100- and 300-ppm exposure groups, reaching up to 100% in the latter group (Fig. 8D).

DISCUSSION

In this research, we sought answers to three questions. For the first question regarding the equivocal induction of HPNs at