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Benzene Inhalation Produces Leukemia in Mice[1,2]

Benzene Inhalation Produces Leukemia in Mice. CRONKITE, E. P., BULLIS, J. E., INOUE, T., AND DREW, R. T. (1984). *Toxicol. Appl. Pharmacol.* 75, 358–361. Female C57Bl/6 mice were exposed to 300 ppm benzene 6 hr/day, 5 days/week for 16 weeks and then held for lifetime observation. Sixty-four weeks after commencement of the study, 10 of 90 exposed mice had died as opposed to only 1 of 88 controls. Of the 10 exposed mice that died, 6 had thymic lymphomas, 2 had unspecified lymphomas, 1 was killed when moribund and found leukemia-free, and 1 was undiagnosed due to autolysis and partial cannibalization. The single dead control animal did not have lymphoma or leukemia. These data provide proof of the leukemogenicity of benzene in female C57Bl/6 mice.

Benzene inhalation in man has been associated with the induction of bone marrow hypoplasia and an increased incidence of acute myeloblastic, promyelocytic, erythroblastic, and lymphoblastic leukemias (Aksoy *et al.*, 1972, 1974, 1976; Vigliani and Forni, 1976). Whether marrow hypoplasia and recovery is a necessary prelude to the induction of leukemia has not been established in man or lower mammals.

There are several reports describing attempts to induce leukemia in rodents (Maltoni and Scarnato, 1979; Snyder *et al.*, 1977; Snyder *et al.*, 1980; Green *et al.*, 1981a; Lerum, 1973; Stoner *et al.*, 1980) or changes in hematopoietic stem cells (Frash *et al.*, 1976; Irons *et al.*, 1979; Johnston *et al.*, 1979; Gill *et al.*, 1980; Tice *et al.*, 1980; Tunek *et al.*, 1981; Harigaya *et al.*, 1981; Cronkite *et al.*, 1982; Pfeifer and Irons, 1982) by either inhalation or ingestion of benzene. The reports on leukemia have been negative or debatable and those on hemopoietic effects uniformly positive. Snyder *et al.* (1980) demonstrated that lifetime exposure to 300 ppm 6 hr/day, 5 days/week, induces an increase in leukemia in C57Bl/6J male mice but not in AKR mice. However, the numbers are small and the significance is debatable. Results of most other studies are equivocal or negative (Ward *et al.*, 1975; Lerum, 1973; Stoner *et al.*, 1980; Harigaya *et al.*, 1981). Inhalation of benzene causes striking effects on lymphocytes (Green *et al.*, 1981b; Cronkite *et al.*, 1982; Pfeifer and Irons, 1982) stem cells (Green *et al.*, 1981b; Frash *et al.*, 1976; Gill *et al.*, 1980; Tunek *et al.*, 1981; Harigaya *et al.*, 1981) and marrow cell kinetics (Irons *et al.*, 1979; Johnston *et al.*, 1979; Tice *et al.*, 1980). We have been investigating effects of concentration, duration of exposure, and recovery time on benzene-induced depression of lymphocytes in blood and/or pluripotent stem cells in bone marrow. This report describes observations made in a group of mice exposed to benzene for 16 weeks and then set aside for lifetime observations.

METHODS

Animals. C57Bl/6 (Brookhaven National Laboratory; BNL) female mice were maintained at 22 ± 2°C and were allowed feed (Purina) and water *ad libitum* except during exposure. A 12-hr on, 12-hr off light cycle was maintained.

Exposures. When mice were 7 to 9 weeks of age, exposure to 300 ppm benzene began (6 hr/day, 5 days/

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[2] Research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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week) and was continued for 16 weeks. A parallel control group of mice was housed in the same room and sham exposed to air in a similar chamber. There were 118 and 116 mice in the exposed and nonexposed groups, respectively. These were reduced to 90 and 88, respectively, by killing 5 mice for hematopoietic stem cell assays at 3, 28, 56, 112, and 168 days after the last exposure and 3 mice after 179 days.

Exposures were carried out in 27-in. stainless-steel and Lucite chambers, similar to those described by Hinners *et al.* (1968). The mice received a total of 78 exposures with the final average integrated concentration being 299.8 ppm. Benzene was generated from a simple bubbler, and monitored routinely by gas chromatography. A wet chemical method was used to verify the gas chromatograph analysis. The benzene was identified as "reagent grade" "Thiophene free" (Mallinckrodt Chemical, Paris, Ky.).

Necropsy and diagnosis of lymphoma–leukemia. If mice appeared ill they were placed in individual cages and blood was drawn for red, white, and differential blood cell counts. However, most animals died before blood counts were made. All mice found dead were immediately injected ip with 2 ml of 10% Formalin–70% alcohol. If it were not possible to necropsy the mice immediately, the abdominal and thoracic cavities were opened from symphysis pubis to the mandible and the carcass placed in buffered 10% Formol and autopsied later.

At autopsy, the general appearance of the thymus, lungs, spleen, liver, and kidneys were noted. These organs as well as bone marrow were fixed in buffered Formalin, stained with hematoxylin and eosin, and evaluated histologically. Criteria used to diagnose hematologic diseases included: red, white, and differential cell counts, enlarged thymus, lymphadenopathy, and splenomegaly. Microscopic criteria include the loss of cortical medullary structure of the thymus with the appearance of a single cell type; partial or complete replacement of the mixed erythrocytic, granulocytic, and megakaryocytic cellular pattern in bone marrow by a single cell type; replacement of white and red pulp of the spleen by a single cell type; the presence of cellular infiltrates of lymphoma cells in liver and kidneys; and infiltration of lymphoma cells into the muscle adjoining bone without producing an inflammatory response.

All of the above criteria are not necessarily present for diagnosis of lymphoma. We considered an enlarged thymus with destruction of the normal structure and the presence of a single lymphoid cell type sufficient for diagnosis of thymic lymphoma. Thymic lymphoma is generally associated with an enlarged spleen, infiltrations of the liver and kidneys, and replacement of the bone marrow. In the absence of thymic enlargement, an enlarged but abnormal spleen with lymphoid cells replacing normal cells in spleen, marrow, and one or more lymph nodes is cause for a diagnosis of lymphoma.

RESULTS

Figure 1 shows the mortality and incidence of lymphoma–leukemia up to 48 weeks after termination of exposure. The upper part of the figure shows the deaths through that period. The lower half shows the histologically confirmed cases of thymic lymphoma, lymphoma, or leukemia. The difference of 10 deaths compared to 8 diagnosed lymphoma–leukemias is accounted for by 1 carcass being lost via cannibalism and autolysis of tissues and a second animal being killed when moribund, but found to be leukemia-free. Of the six mice diagnosed as having thymic lymphoma five had enlarged thymuses, spleen, and lymph nodes. They also had infiltrates of lymphoid cells in bone marrow, spleen, thymus, lymph nodes, liver, and kidneys. One had an enlarged thymus with one cell type and hepatic infiltrates of lymphoid cells, but no adenopathy or splenomegaly. The two mice which were diagnosed as having lymphoma had splenomegaly and lymphadenopathy with typical lymphoid replacement of normal lymph nodes, splenic structure, and bone marrow. One control mouse was killed moribund at 270 days after termination of exposure and had no histologic evidence of lymphoma–leukemia. It had a white blood cell count of 4500/mm^3 and differential of 25% segmented neutrophils, 69% lympho-

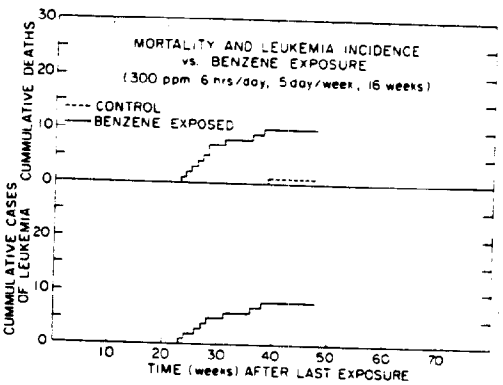

FIG. 1. Cumulative deaths and leukemia after exposure to benzene.

cytes, 6% monocytes, typical of control mice of this age and strain. Death rates due to lymphoma–leukemia were compared for 118 benzene-exposed mice and 354 recent historical controls of this sex and strain from this laboratory by a Cox model of survival analysis (Cox, 1972) with benzene exposure as a covariate. A log-likelihood test indicated that benzene exposure is a significant variable ($p < 0.0001$) in predicted lymphoma–leukemia death rate.

DISCUSSION

The C57Bl/6 mouse harbors a radiation leukemia virus (Lieberman and Kaplan, 1959). When they are exposed to 175 rad at weekly intervals for 4 weeks, a very high incidence of thymic lymphoma is observed (Kaplan and Brown, 1952). In current unpublished studies on C57Bl/6 BNL,[3] three exposures of 175 rad, each 8 days apart, produced a significant depression in bone marrow cellularity and the hematopoietic stem cell population. Previous studies in our laboratory showed that 16 weeks of exposure to benzene also produces a major perturbation in the marrow cellularity and depression of the stem cell population. Sixteen weeks after the last exposure, the number of stem cells was still only 58.5% of that in controls, however, they had recovered to 92% of control values by 25 weeks. In contrast the blood lymphocytes returned to normal levels by 8 weeks after the last exposure (unpublished observations).

This study differs from most benzene leukemogenesis studies in which exposures were continued for the life of the rodents. Our study allowed for hemopoietic recovery without further benzene exposure. The incidence of lymphoma–leukemia 64 weeks after beginning exposure is 8% in benzene-exposed mice compared to 0% in the control mice. Moreover, all cases of leukemia occurred in the period from 23 to 38 weeks after final exposure in the benzene group. One control mouse was killed when moribund. Histologic study did not reveal any evidence of lymphoma–leukemia or the cause of its illness.

Whether benzene has decreased the latent period for development of lymphoma–leukemia or increased the absolute incidence cannot be determined until completion of this lifetime study. We present these preliminary data because of the intense interest in toxicity of benzene in the environment and workplace.

Further studies are required to establish whether benzene is acting directly as a leukemogen or through activation of the radiation leukemia virus as a result of the necrosis and regeneration of primitive hemopoietic cells harboring the virus. In view of the probably positive leukemogenic results of Snyder *et al.* (1980) following lifetime exposure to benzene and our positive results, with 16 weeks of exposure at the same time-concentrations of benzene in C57Bl/6 mice, studies on the relationship of concentration and exposure time on leukemogenesis are required for investigation of the mechanism by which benzene induces lymphoma–leukemia. Such studies should include a systematic determination of whether perturbation of hemopoiesis is a necessary prelude to the development of leukemia.

[3] It is assumed but not proven that our C57Bl/6 mice inbred at BNL harbors the same radiation leukemia virus.

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