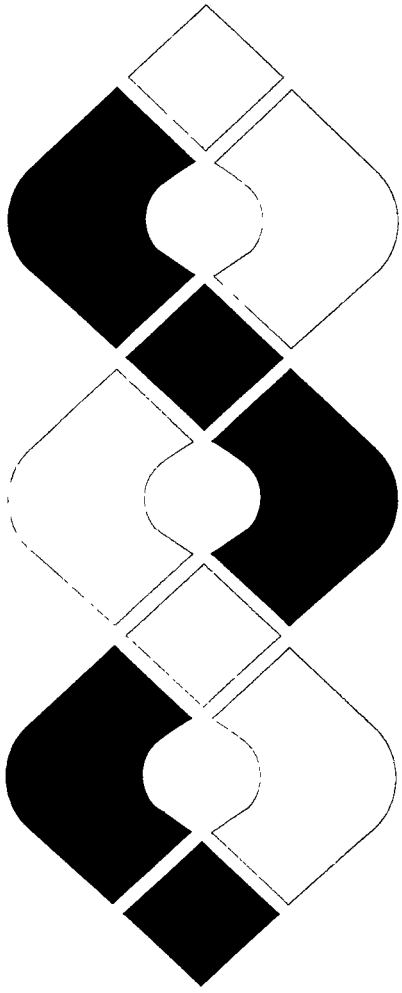
PNL-10500 Pt. 1
UC-408

Pacific Northwest Laboratory Annual Report for 1994 to the DOE Office of Energy Research

Part 1: Biomedical Sciences
April 1995



Prepared for the U.S. Department of Energy
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Pacific Northwest Laboratory Annual Report for 1994 to the DOE Office of Energy Research

Part 1: Biomedical Sciences

J. F. Park and Staff

April 1995

Prepared for
the U.S. Department of Energy
under Contract DE-AC06-76RLO 1830

Pacific Northwest Laboratory
Richland, Washington 99352

Preface

This 1994 Annual Report from Pacific Northwest Laboratory (PNL) to the U.S. Department of Energy (DOE) describes research in environment and health conducted during fiscal year (FY) 1994. This year, the report consists of two parts, each in a separate volume.

The two parts of the report are oriented to particular segments of the PNL program, describing research performed for the DOE Office of Health and Environmental Research (OHER) in the Office of Energy Research. Each part consists of project reports authored by scientists from several PNL research departments, reflecting the multidisciplinary nature of the research effort.

The parts of the 1994 Annual Report are as follows:

Part 1: Biomedical Sciences	J.F. Park, Program Manager F.T. Cross, Report Coordinator C.C. Lumetta, Editor
Part 2: Atmospheric Sciences	W.R. Barchet, Program Manager G.P. O'Connor, Editor

Activities of the scientists whose work is described in this annual report are broader in scope than the articles indicate. PNL staff have responded to numerous requests from DOE during the year for planning, for service on various task groups, and for special assistance.

Credit for this annual report goes to the many scientists who performed the research and wrote the individual project reports, to the program managers who directed the research and coordinated the technical progress reports, to the editors who edited the individual project reports and assembled the two parts, and to Ray Baalman, editor in chief, who directed the total effort.

Previous Annual Reports in this series:

1951	HW-25021, HW-25709
1952	HW-27814, HW-28636
1953	HW-30437, HW-30464
1954	HW-30306, HW-33128, HW-35905, HW-35917
1955	HW-39558, HW-41315, HW-41500
1956	HW-47500
1957	HW-53500
1958	HW-59500
1959	HW-63824, HW-65500
1960	HW-69500, HW-70050
1961	HW-72500, HW-73337
1962	HW-76000, HW-77609
1963	HW-80500, HW-81746
1964	BNWL-122
1965	BNWL-280, BNWL-235, Vol. 1-4; BNWL-361
1966	BNWL-480, Vol. 1; BNWL-481, Vol. 2, Pt. 1-4
1967	BNWL-714, Vol. 1; BNWL-715, Vol. 2, Pt. 1-4
1968	BNWL-1050, Vol. 1, Pt. 1-2; BNWL-1051, Vol. 2, Pt. 1-3
1969	BNWL-1306, Vol. 1, Pt. 1-2; BNWL-1307, Vol. 2, Pt. 1-3
1970	BNWL-1550, Vol. 1, Pt. 1-2; BNWL-1551, Vol. 2, Pt. 1-2
1971	BNWL-1650, Vol. 1, Pt. 1-2; BNWL-1651, Vol. 2, Pt. 1-2
1972	BNWL-1750, Vol. 1, Pt. 1-2; BNWL-1751, Vol. 2, Pt. 1-2
1973	BNWL-1850, Pt. 1-4
1974	BNWL-1950, Pt. 1-4
1975	BNWL-2000, Pt. 1-4
1976	BNWL-2100, Pt. 1-5
1977	PNL-2500, Pt. 1-5
1978	PNL-2850, Pt. 1-5
1979	PNL-3300, Pt. 1-5
1980	PNL-3700, Pt. 1-5
1981	PNL-4100, Pt. 1-5
1982	PNL-4600, Pt. 1-5
1983	PNL-5000, Pt. 1-5
1984	PNL-5500, Pt. 1-5
1985	PNL-5750, Pt. 1-5
1986	PNL-6100, Pt. 1-5
1987	PNL-6500, Pt. 1-5
1988	PNL-6800, Pt. 1-5
1989	PNL-7200, Pt. 1-5
1990	PNL-7600, Pt. 1-5
1991	PNL-8000, Pt. 1-5
1992	PNL-8500, Pt. 1-4
1993	PNL-9000, Pt. 1-4
1994	PNL-10500, Pt. 1-2

Foreword

This report summarizes FY 1994 progress in biological and general life sciences research programs conducted for the Department of Energy's Office of Health and Environmental Research (OHER) at Pacific Northwest Laboratory (PNL). This research provides knowledge of fundamental principles necessary to identify, understand, and anticipate the long-term health consequences of exposure to energy-related radiation and chemicals. Our emphasis is to understand the mechanisms involved in radiation- and chemically induced damage. Through this understanding, the health risks associated with exposure to effluents from energy-related technologies can be better defined, and the uncertainty associated with those risks decreased.

The sequence of this report of PNL research reflects the OHER programmatic structure. The **Biological Research** section contains reports of research including studies of the impact of radiation, radionuclides, and chemicals on biological responses at all levels of biological organization. The **General Life Sciences Research** section reports research conducted for the OHER human genome program.

Biological Research

The progress in several life-span studies in rats and dogs on the effects of inhaled radioactive materials including radon, $^{238}PuO_2$, $^{239}PuO_2$, and $^{239}Pu(NO_3)_4$ is reviewed. Recent progress describes exposure/dose-effect relationships for radon-induced lung tumors and plutonium-induced tumors of the beagle liver and skeleton.

Because many of the life-span studies using experimental animals are in the late phase of completion, it is essential to ensure that we do not lose valuable data or experimental materials generated by these studies. To this end, we have been conducting a comprehensive effort to gather, organize, and catalog data, documents, and tissues related to life-span radiobiology studies for future research and analyses. A related new project reports on progress made toward developing standardized analyses of data from all DOE life-span animal studies.

The animal studies on cancer induction from radon continue to be the core of an extensive research program that characterizes each step in the exposure–dose–response pathway of radon. We are combining in vivo and in vitro methods with up-to-date exposure systems and modern cytogenetic and molecular techniques to understand the mechanisms of radon carcinogenesis, and to reduce the uncertainty in extrapolating molecular/cellular/animal data to risk assessment in mine and home environments.

The relationship between site and type of initial DNA damage and the development of molecular changes, mutations, chromosome damage, and cell transformation contributes to an understanding of the disease process. Chemical- and radiation-effects studies are being conducted using molecular techniques to understand the sites of radiation-induced damage, the types of products produced, and the binding of carcinogenic chemicals to the DNA. The influence that primary, secondary, and tertiary DNA structure has on the induction and location of DNA damage and binding is being determined.

Laboratory-Wide Effort

Biomedical research at PNL is an interdisciplinary effort requiring scientific contributions from many research departments throughout the laboratory. Personnel in the Life Sciences Center are the principal contributors to this report.

Additional information on PNL research efforts can be obtained by requesting reprints from the list of publications found at the back of this report.

Contents

Preface iii

Foreword v

Biological Research

Inhaled Plutonium in Dogs, J.F. Park 1
National Radiobiology Archives, C.R. Watson 11
Statistical Analysis of Data from Radiobiologic Animal Studies, E.S. Gilbert 19
Genotoxicity of Inhaled Energy Effluents, A.L. Brooks 23
Molecular Events During Tumor Initiation, D.L. Springer 29
Biochemistry of Free Radical-Induced DNA Damage, A.F. Fuciarelli 35
Radon Hazards in Homes, F.T. Cross 41
Genetics of Radon-Induced Lung Cancer, J.D. Saffer 47
Mechanisms of Radon Injury, R.F. Jostes 53
In Vivo/In Vitro Radon-Induced Cellular Damage, A.L. Brooks 57

Publications and Presentations

Publications 63
Presentations 69

Author Index 79
Distribution Distr.1

Biological Research

Inhaled Plutonium in Dogs

Principal Investigator: J.F. Park

Other Investigators: R.L. Buschbom, G.E. Dagle, E.S. Gilbert, C.R. Watson, and R.E. Weller

These projects (Inhaled Plutonium Oxide in Dogs, Inhaled Plutonium Nitrate in Dogs) are life-span dose-effects studies of inhaled $^{239}PuO_2$, $^{238}PuO_2$, and $^{239}Pu(NO_3)_4$ in beagles. This report describes work performed in FY 1994 to study preliminary life-span dose-effect relationships in the skeleton and liver of beagles given single exposures of $^{239}Pu(NO_3)_4$ aerosols to obtain dose level groups of 20 dogs with initial lung depositions of 63, 11, 2.2, 0.32, or 0.12 kBq. Following life-span observation, the skeleton contained 48% of the final body deposition of plutonium, and the liver 40%. Significant life-span shortening was observed in the 3 highest exposure levels, with bone tumors occurring at a group exposure level of 63 Bq lung deposition, and bone and liver tumors occurring at group average exposure levels of 11 and 2.2 kBq lung deposition. The 37 bone tumors (in 28 dogs) were primarily in the axial skeleton (62% compared with 38% for appendicular skeleton). Elevated serum liver enzyme levels were observed in groups with mean liver plutonium concentrations of 1.3 Bq g^{-1} and mean average liver doses of 31 cGy or greater. Adenomatous hyperplasia and bile-duct hyperplasia were also observed. A total of 19 primary liver tumors occurred in 14 plutonium-exposed dogs at lower exposure levels, where life span was not shortened by the occurrence of bone (or lung) tumors.

The life-span biological effects of inhaled plutonium were studied in beagles to help predict risks of effects from accidental exposure in people (Table 1). All the dogs are dead and radioanalysis of tissues completed. Review of dosimetry and dose-effects observations, including histopathology, occurred this year in conjunction with the preparation of manuscripts. The *Pacific Northwest Laboratory Annual Report for 1989 to the DOE Office of Energy Research,* Part 1, summarized the results of the $^{239}PuO_2$ study, and the results of the $^{238}PuO_2$ study were summarized in the *Annual Report for 1990.*

These life-span beagle studies are part of a multilaboratory program to evaluate several radionuclides and routes of exposure. In our studies the plutonium was administered by inhalation, and lung tumors were a frequent biological effect. Bone, liver, and lymph node lesions were observed following plutonium translocation to those organs. Preliminary results on the effects of inhaled $^{239}Pu(NO_3)_4$ on the skeleton and liver are presented in this report.

Life-span shortening was observed in dogs exposed to inhaled $^{239}Pu(NO_3)_4$ at the 3 highest exposure levels (Figure 1). Dogs at the highest exposure level died early from radiation pneumonitis and are not discussed further in this report. At necropsy of the remaining dogs, the skeleton contained 48% of the final body deposition of plutonium, and the liver 40%; the liver-to-skeleton ratios were similar at each exposure level and time post exposure, and without regard to final body depositions and sex of dog.

Plutonium is translocated to the skeleton (Figure 2) fitting an uptake curve of

$$S(t) = 0.350e^{-0.000014t} - 0.142e^{-0.0029t} - 0.208e^{-0.24t}$$

where S(t) is the proportion of the initial lung deposition (ILD) in the skeleton at time t (days post exposure). The equation used for calculating dose to the skeleton at time t was

Table 1. Exposure/Dose Protocols in Life-Span Studies with Inhaled Plutonium in Dogs

Exposure-Level Group	Number of Dogs Male	Number of Dogs Female	Initial Lung Deposition[a] kBq[b] (total)	Initial Lung Deposition[a] Bq/g Lung[b] (concentration)
		$^{239}PuO_2$ (Exposed 1970 and 1971)		
Control	10	10	0	0
1	10	11	0.12 ± 0.05	0.93 ± 0.39
2	11	11	0.69 ± 0.14	6.2 ± 1.3
3	11	10	2.7 ± 0.05	23 ± 4
4	12	12	11 ± 2	95 ± 17
5	10	10	41 ± 6	349 ± 46
6	3	5	213 ± 120	2130 ± 1160
	67	69		
		Inhaled $^{238}PuO_2$ (Exposed 1973 and 1974)		
Control	10	10	0	0
1	10	10	0.082 ± 0.031	0.65 ± 0.24
2	11	10	0.67 ± 0.12	5.9 ± 1.2
3	12	10	2.9 ± 0.4	24 ± 3
4	10	10	13 ± 3	107 ± 22
5	10	10	52 ± 10	408 ± 72
6	7	6	203 ± 54	1737 ± 440
	70	66		
		Inhaled $^{239}Pu(NO_3)_4$ (Exposed 1976 and 1977)		
Control	10	10	0	0
Vehicle	10	10	0	0
1	10	10	0.12 ± 0.04	0.97 ± 0.38
2	10	10	0.32 ± 0.06	2.6 ± 0.5
3	10	10	2.2 ± 0.3	19 ± 4
4	10	10	11 ± 1	91 ± 15
5	10	10	63 ± 11	520 ± 107
6	3	2	202 ± 84	1772 ± 747
	73	72		

(a) Estimated from external thorax counts at 2 and 4 weeks after exposure, and from estimated lung weights (0.011 x body weight).
(b) Mean ± 95% confidence intervals around mean.

$$d_t = (KAE/m) \int_0^t S(t)dt$$

where d_t is the cumulative dose at time t (Gy); K is 1.38 x 10^{-5}, conversion factor for converting Bq to Gy; E is 5.15 MeV, energy of ^{239}Pu alpha-particle emission; A is ILD (Bq); m is skeleton weight (g) = (0.1) [body weight at exposure (g)]; t is time after exposure (d); and S(t) is skeleton retention function.

The microdistribution of plutonium in bone, using neutron-induced fission tracks, from 1 dog in this study was compared with 1 dog intravenously injected (at the University of Utah) with plutonium citrate at a similar dose. The plutonium concentration on the trabecular and periosteal surfaces was a factor of about 2 higher for the injection route compared to the inhalation route.

Bone tumors were the main cause of death or euthanasia in the 2 highest exposure levels discussed in this report [63 or 11 kBq ILD

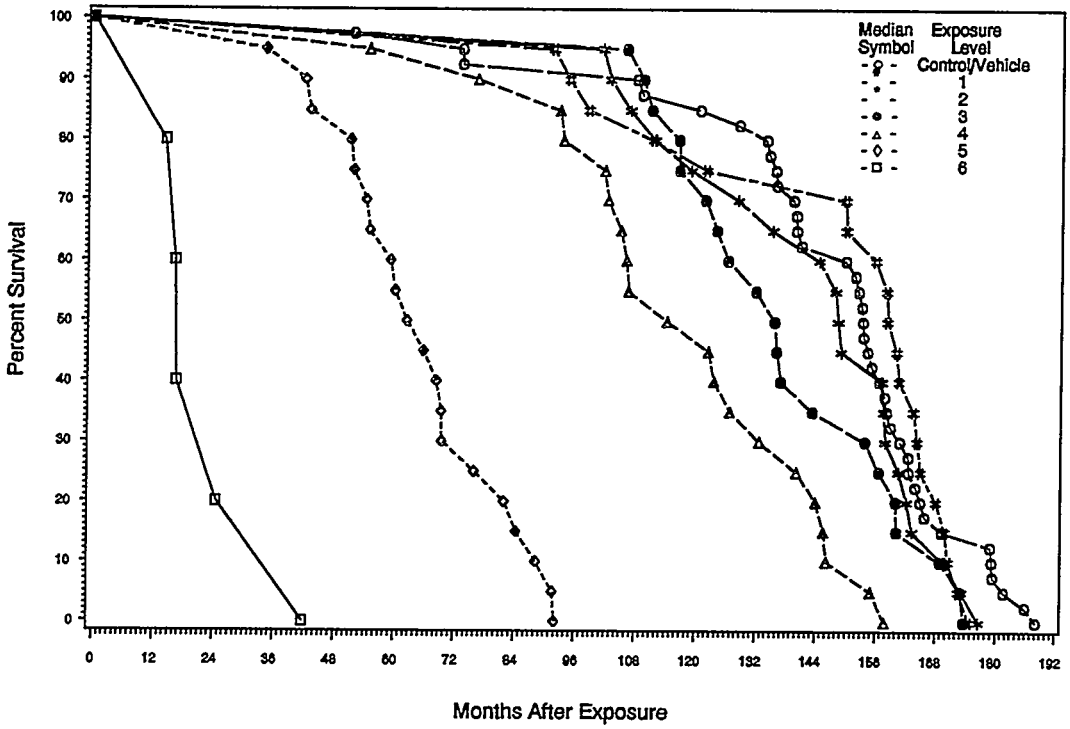

Figure 1. Life-Span Shortening of Dogs Exposed to $^{239}Pu(NO_3)_4$

(Table 2)]. The tumors were osteogenic sarcomas arising from endosteal surfaces, except for 2 hemangiosarcomas in vertebra of 2 dogs from group 2, and an anaplastic osteosarcoma in the radius of 1 dog from group 3. Metastases occurred to the lungs of 10 dogs, kidneys of 4 dogs, regional lymph nodes of 2 dogs, and occasionally other systemic sites. Peritrabecular fibrosis was observed as a deterministic finding in all groups that produced bone tumors. The tumors arose primarily in the axial skeleton, with a similar distribution of bone tumors as occurred following inhalation of $^{238}PuO_2$ (Table 3). It should be noted that the tumors per estimated number of lining cells was similar for the axial and appendicular skeletons.

Bone-tumor risk was calculated for the purposes of comparing our data with that reported from dogs intravenously injected with plutonium citrate at the University of Utah (Mays et al. 1987). Using only incidence data unadjusted for competing causes of death, our bone-tumor incidence fit the following quadratic curve:

$$BTI = (99.3 \pm 4.9)D - (29.4 \pm 3.3)D^2$$

where BTI is the bone-tumor incidence and D is the skeletal dose (cGy) 1 year prior to death. Our bone-tumor incidence was similar to that reported at the University of Utah (Figure 3), but it should be noted that we were not addressing the question of threshold or the shape of the dose-response curve at very low exposure levels.

Plutonium accumulations in the liver were described with a 3-compartment model as follows:

$$L(t) = 0.29e^{-0.000035t} - 0.165e^{-0.0029t} - 0.125e^{-0.24t}$$

where L(t) is the proportion of ILD in the liver at time t (days post exposure). The retention curves were not statistically different between exposure levels, or between male or female dogs.

The mean liver doses (Table 2) calculated by integrating from the day of exposure to 1 year prior to death [to compare our findings with those from other beagle studies with intravenously injected plutonium (Taylor et al.

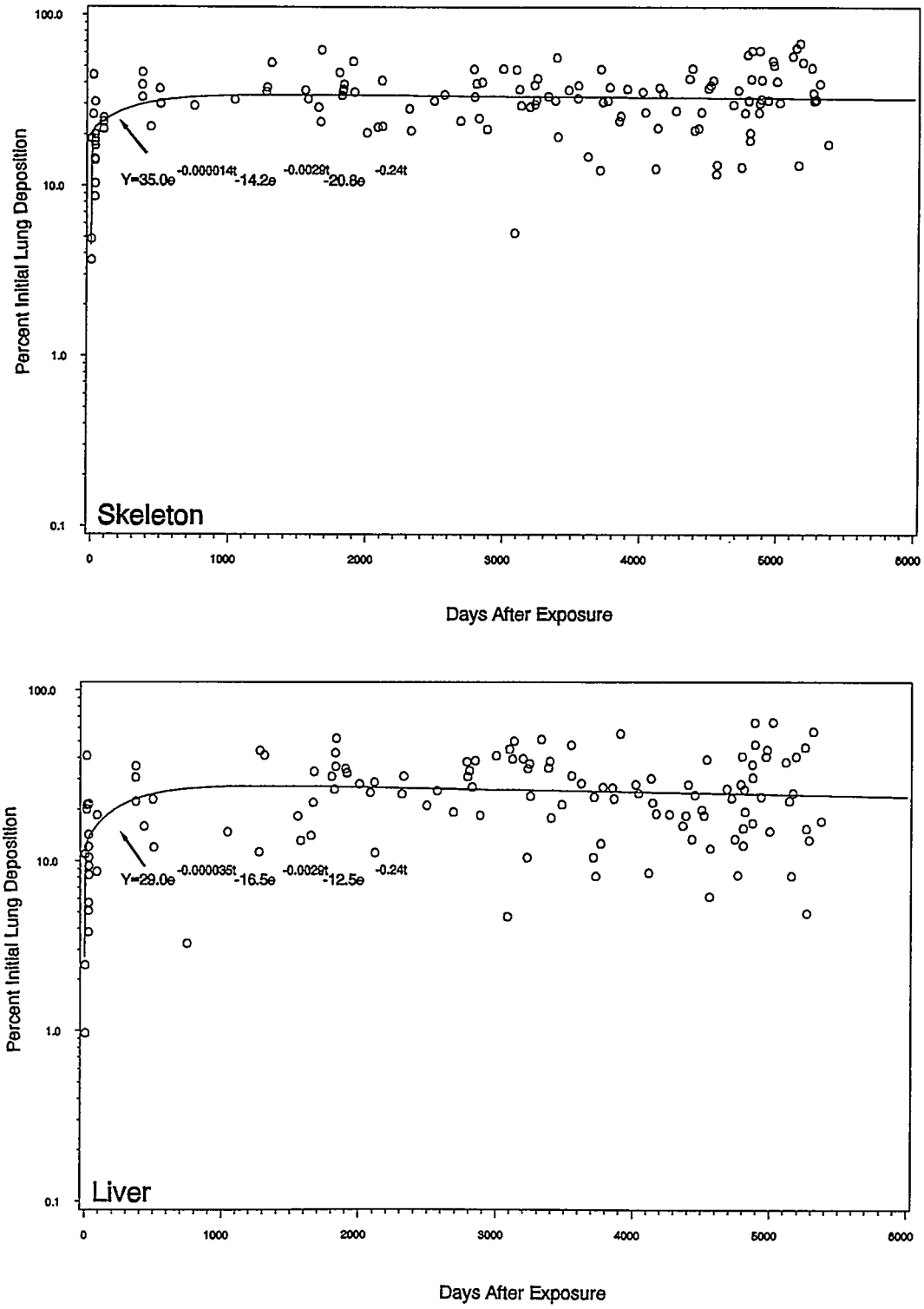

Figure 2. Plutonium Distribution in Dogs Exposed to $^{239}Pu(NO_3)_4$

Table 2. Skeleton and Liver Effects Data in Dogs Exposed to $^{239}Pu(NO_3)_4$[a]

Exposure Group	5	4	3	2	1	Vehicle	Control
Study Summary							
Number of dogs	20	20	20	20	20	20	20
Initial lung deposition (kBq)	63 ± 5	11 ± 1	2.2 ± 0.14	0.32 ± 0.03	0.12 ± 0.02	--	--
Initial lung concentration (Bq/g)	520 ± 51	91 ± 7	19 ± 2	2.6 ± 0.3	0.98 ± 0.18	--	--
Median survival (mo)	62	114	135	157	150	154	139
Skeleton							
Deposition (kBq)	23.4 ± 3	3.4 ± 0.3	0.72 ± 0.05	0.10 ± 0.01	0.05 ± 0.01	--	--
Concentration (Bq/g)	15.8 ± 2.1	2.1 ± 0.2	0.49 ± 0.06	0.07 ± 0.01	0.03 ± 0.01	--	--
Dose[b] (cGy)	190 ± 15	71 ± 5	20 ± 3	2.7 ± 0.3	1.1 ± 0.2	--	--
Tumor incidence	85	50	5	0	0	0	0
Liver							
Deposition (kBq)	16.1 ± 2	2.9 ± 0.3	0.49 ± 0.04	0.08 ± 0.01	0.04 ± 0.01	--	--
Concentration (Bq/g)	61.0 ± 8.5	6.9 ± 0.9	1.3 ± 0.12	0.24 ± 0.04	0.12 ± 0.04	--	--
Dose[b] (cGy)	296 ± 24	111 ± 7	31 ± 4	4.2 ± 0.5	1.7 ± 0.3	--	--
Adenomatous Hyperplasia							
Incidence	17	18	19	19	16	14	15
Average severity[c]	2.0	2.8[d]	2.7[d]	2.7[d]	2.0	1.8	1.7
Bile-Duct Hyperplasia							
Incidence	1	8	12	10	15	6	10
Average severity[c]	0.1	0.7	0.9	0.8	1.4[d]	0.4	0.6
Liver Tumors							
Dogs with liver tumor	0	5	3	3	3	0	1
Cholangioadenoma	0	4	2	0	0	0	0
Cholangiocarcinoma	0	4	1	1	2	0	0
Hepatocellular carcinoma	0	1	0	2	1	0	1
Leiomyosarcoma	0	1	0	0	0	0	0
Average age at death of dogs with liver tumors (mo)	--	136	146	151	171	--	162
Percent dogs with liver tumors	0	25[e]	15	15	15	0	5

(a) Values shown are mean ± standard error.
(b) Dose accumulated to 1 year prior to death.
(c) If condition was observed, severity was graded from 1 to 5, average based on n = incidence
(d) Dose-group mean significantly higher than the control group mean ($P<0.05$)
(e) Dose-group tumor incidence significantly higher than the tumor incidence for vehicle and control combined ($P<0.05$)

Table 3. Bone Tumors in Dogs Inhaling Plutonium: Distribution in Different Bones

	^{239}Pu Nitrate		^{238}Pu Oxide		Total		% Weight [a]	Lining Cells X10^{7}[b]	Tumors per 10^{7} Lining Cells
	No. Tumors	% Tumors	No. Tumors	% Tumors	No. Tumors	% Tumors			
Axial Skeleton									
Skull and maxilla	2	5	1	3	3	4	13.4	55.1	0.05
Mandible	1	3	0	0	1	1	4.5	30.8	0.03
Cervical vertebrae	4	11	2	5	6	8	5.6	34.9	0.17
Thoracic vertebrae	4	11	5	13	9	12	6.7	48.1	0.19
Lumbar vertebrae (including sacrum)	9	24	9	23	18	23	5.7	37.9	0.47
Coccygeal vertebrae	0	0	0	0	0	0	2.8	--	--
Sternum	0	0	1	3	1	1	1.3	--	--
Ribs (with cartilage)	3	8	5	13	8	10	10.4	38.1	0.21
Total/Average	**23**	**62**	**23**	**58**	**46**	**60**	**50.4**	--	**0.19**
Appendicular Skeleton									
Scapula	1	3	5	13	6	8	4.3	20.3	0.30
Humeri	6	16	1	3	7	9	7	43.1	0.16
Radii/ulna	1	3	0	0	1	1	5.7	25	0.04
Forepaws	0	0	0	0	0	0	4.4	--	--
Pelvis	4	11	7	18	11	14	6.3	26.6	0.41
Femora	2	5	2	5	4	5	7.3	45.3	0.09
Tibiae/fibulae	0	0	2	5	2	3	6.9	33.4	0.06
Hindpaws	0	0	0	0	0	0	7.7	--	--
Total/Average	**14**	**38**	**17**	**43**	**31**	**40**	**49.6**	--	**0.18**
Total	**37**	**100**	**40**	**100**	**77**	**100**	**100**	--	--

(a) Raabe and Parks 1993.
(b) Polig and Jee 1989.

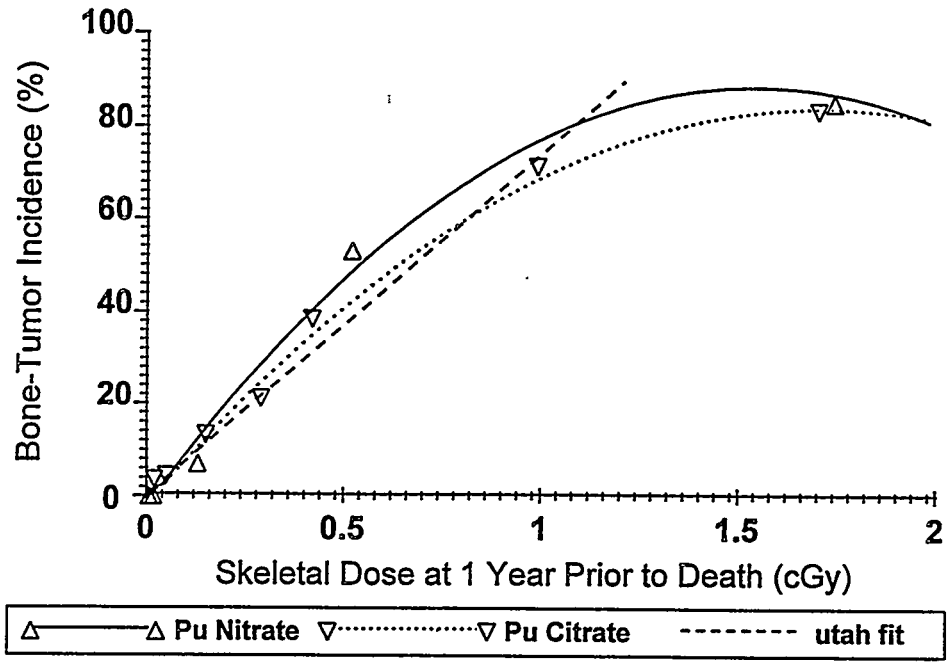

Figure 3. Bone-Tumor Incidence in Dogs Inhaling $^{239}Pu(NO_3)_4$ Compared with Mays et al. (1987) Plutonium Citrate Study

1991)], and multiplying by the appropriate factors to convert to dose in Gy. ILD used in the dose calculation was estimated from external thorax counts at 14 days after exposure; if the estimated ILD was less than the final body deposition, the final body deposition was used. A liver weight (with blood) of 5% of the dog weight at exposure was used for dose calculations.

Autoradiography of the liver from 3 dogs showed single alpha tracks deposited primarily over liver parenchymal cells and occasionally some aggregates in vacuolated sinusoidal cells. When the location of the tracks were plotted against the relative distance from the portal area to the central vein, there was a tendency for the alpha tracks to be present primarily in the midzonal areas. Calculations (made by counting alpha tracks) of radioactivity concentration in autoradiographs gave about a 2-fold higher activity in the parenchymal (and sinusoidal cells) than determined by radioanalysis of the whole organ, because radioanalysis of the whole organ included tissues without plutonium deposition. The occurrence of plutonium primarily in single tracks in the livers of dogs exposed to $^{239}Pu(NO_3)_4$ contrasted with the large alpha stars (indicating particles) translocated in the $^{239}PuO_2$-exposed dogs and both single alpha tracks and alpha stars in the $^{238}PuO_2$-exposed dogs.

Liver damage was evaluated by observing serum levels of liver enzymes. Elevated serum chemistry values first occurred at 4.1 years post exposure in group 5 dogs when the group average accumulative liver dose was 2.8 Gy. At this time, the dose rate was 60 to 70 cGy per year. There were biphasic elevations of serum alkaline phosphatase (ALP) and serum glutamic pyruvic transaminase (GPT) in individual dogs. An early increase was followed by a return to control values, and a later effect was characterized by persistent, increased elevations of both ALP and GPT. Values for GPT and ALP in groups 3 and 4 also became significantly higher ($P \leq 0.05$) than those for the control group (Figure 4).

Degenerative liver lesions became more common as the dogs aged. The group average mean grade of adenomatous hyperplasia of liver parenchymal cells was significantly higher

($P \leq 0.05$) in groups 2, 3, and 4 dogs relative to control dogs (Table 2). The adenomatous (nodular) hyperplasia consisted of lobules with parenchymal cells of increased size, increased vacuolation, and decreased lipofuscin content relative to surrounding liver lobules. There was a trend towards increased bile-duct hyperplasia consisting of aggregates of bile ducts in periportal areas and, occasionally, bile ducts extending into liver lobules. This finding generally was not significantly different in the plutonium-exposed dogs compared to the control dogs. There were additional changes not clearly related to plutonium exposures, such as the aggregation of vacuolated sinusoidal cells that increased in incidence and group average severity as the dogs became older.

Liver tumors occurred in groups of dogs living longer than the minimal latency period. A total of 10 intrahepatic bile-duct tumors occurred in 5 dogs from group 4: 4 cholangiocarcinomas (1 was fatal), 4 cholangioadenomas, 1 hepatoma, and 1 leiomyosarcoma. Intrahepatic bile-duct tumors were also present in 3 dogs from group 3, 1 dog from group 2, and 2 dogs from group 1; these consisted of 4 cholangiocarcinomas and 2 cholangioadenomas. In addition, fatal hepatocellular carcinomas occurred in 2 dogs from group 2 and nonfatal hepatocellular carcinomas were present in 1 dog each from group 1 and the control group. The first liver tumor occurred in a group-4 dog 123 months following exposure, and the mean average time from exposure to death for the dogs with liver tumors was lowest in the group-4 dogs. Metastases occurred only with the cholangiocarcinomas; they were widespread in 1 dog from group 4 and found in the hepatic lymph nodes or spleen of 1 dog each from groups 3 and 4. Of the 14 plutonium-exposed dogs with liver tumors, 9 were males and 5 were females; the control dog was a female. When comparing the groups of dogs exposed to plutonium with the 2 control groups, the tumor incidence was significantly ($P \leq 0.05$) higher in dogs from group 4 than the tumor incidence for vehicle and control dogs combined.

The liver-cancer risk was difficult to assess due to the low number of tumors and the competing risks of life shortening from lung and bone tumors. We estimated approximately 900 liver tumors per 10^4 dogs per Gy accumulative dose determined from radioanalysis of tissues, assuming a linear dose response in the lower exposure levels. At these lower exposure levels (1 through 4) the liver-tumor dose-response curve that best fit the data was

$$y = (14.01 \pm 1.04) + (0.094 \pm 0.018)x$$

where y = liver-tumor incidence (%) and x = liver dose at 1 year prior to death (cGy). We assumed a linear dose response for these calculations in order to compare our data with the liver tumor risks estimated from dogs intravenously injected with plutonium citrate, where a liver risk estimate of approximately 2000 total liver malignancies per 10^4 dogs per Gy was reported (Taylor et al. 1991).

Caution is in order when inferring the risk of exposure to 1 radionuclide to that of another. While human data are available from Thorotrast patients, it would be inappropriate to derive a plutonium exposure risk from these data, because there are differences in tissue dose distribution for the 2 radionuclides.

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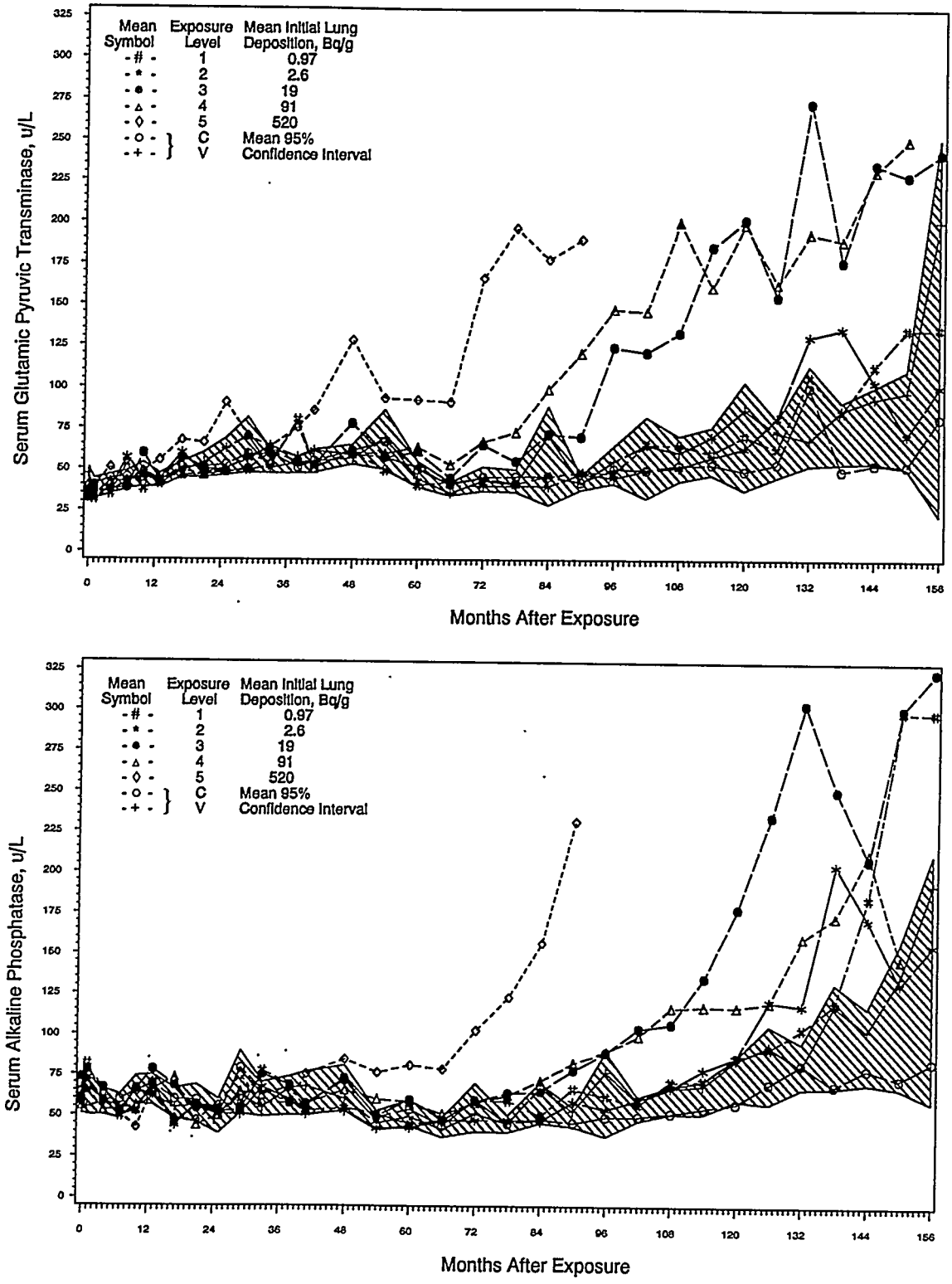

Figure 4. Serum Enzyme Levels in Dogs After Inhaling $^{239}Pu(NO_3)_4$

National Radiobiology Archives

Principal Investigator: C.R. Watson

Other Investigators: S.K. Smith, E.K. Ligotke, J.C. Prather, L.G. Smith, and M.T. Karagianes

The National Radiobiology Archives (NRA) project is a comprehensive effort to gather, organize, and catalog data, documents, and tissues related to completed radiobiology studies. This archiving activity will provide researchers with information for statistical analyses to compare results of these and other studies. The NRA also will provide materials for application of advanced molecular biology techniques to address questions, such as those related to DNA modification, that could not have been considered when these studies were performed. The NRA has a collaborative relationship with the Eurpean Radiobiological Archive (ERA) and the Japanese Low Dose Information Project. The NRA and ERA databases are compatible and can be integrated.

Many investigations have been conducted into the biological effects of ionizing radiation. The focus has been on understanding the nature of human health effects and on quantifying dose–response relationships. When acute effects of large doses had been adequately characterized, attention shifted to effects of lower doses and lower dose rates. This focus led to initiation of life-span studies of experimental animals in several laboratories supported by the US Atomic Energy Commission (AEC), now the Department of Energy (DOE). As DOE radiobiology studies are completed, the National Radiobiology Archives (NRA) will continue to integrate and preserve this unique body of information and materials, and will continue to encourage and simplify its use.

The NRA project concentrated initially on studies of beagle dogs exposed to ionizing radiation at 5 DOE-supported laboratories. The project now includes similar studies using other species and at other laboratories. Three major activities are associated with this project:

1. NRA implements an interlaboratory, computerized *information system* containing a summarized dose-and-effects database, a collection inventory database, and a bibliographic database. During the past year, database structures were redesigned to be compatible with the European Radiobiological Archive (ERA). The information system now includes records from 9 laboratories on approximately 7000 beagle dogs (Table 1), 40,000 rats and mice, and more than 200 nonhuman primates (Table 2). An electronic introduction to the system is available on DOS diskette.

2. NRA establishes a *document archives* of original (or "record copy") research materials such as logbooks, clinical notes, radiographic films, and pathologists' observations. The first major collection of documents and radiographs was transferred from the University of California at Davis (UC Davis) in 1992.

3. NRA establishes a *specimen archives* for research materials such as tissue samples or histopathology blocks and slides. Tissue specimens, histopathology blocks and slides, serial radiographs, and extensive clinical records from more than 1000 dogs from UC Davis are organized and available. Two groups of investigators

Table 1. Major Life-Span Beagle Studies Being Incorporated into the National Radiobiology Archives

NRA Study ID[a]	Dates of Exposures	Description of Study	Number of Life-Span Animals
1-1	1951-1974	^{239}Pu, IV injection	285
1-2	1953-1970	^{226}Ra, IV injection	164
1-3	1954-1963	^{228}Ra, IV injection	89
1-4	1954-1963	^{228}Th, IV injection	94
1-5	1955-1966	^{90}Sr, IV injection	99
1-6	1966-1975	^{241}Am, IV injection	117
1-7	1971-1974	^{249}Cf, IV injection	36
1-8	1971-1973	^{252}Cf, IV injection	35
1-9	1972-1978	^{238}Pu, IV injection (juvenile)	75
1-10	1973	^{253}Es, IV injection	5
1-11	1975-1978	^{238}Pu, IV injection (aged)	34
1-12	1975-1978	^{226}Ra, IV injection (juvenile)	53
1-13	1975-1980	^{226}Ra, IV injection (aged)	33
1-14	1977-1979	^{224}Ra, IV injection (multiple)	128
2-1	1952-1958	X ray, whole body (fractionated)	360
2-2	1961-1969	^{90}Sr, ingested (*in utero* to 540 days)	483
2-3	1964-1969	^{90}Sr, IV injection	45
2-4	1964-1969	^{226}Ra, IV injection (multiple)	335
3-1	1956	^{90}Sr, Transplacental	53
3-2	1957	^{90}Sr, SC injection (multiple, various ages)	98
3-3	1960-1964	^{144}Ce, IV injection	49
3-4	1961-1963	^{137}Cs, IV injection	65
3-5	1968-1978	Gamma ray, whole body (continuous to death)	311
3-6	1968-1977	Gamma ray, whole body (continuous to predetermined dose)	343
4-1	1959-1962	$^{238}PuO_2$, Inhalation	35
4-2	1967	$^{238}PuO_2$, Inhalation	22
4-3	1970-1972	$^{239}PuO_2$, Inhalation	136
4-4	1972-1975	$^{238}PuO_2$, Inhalation	136
4-5	1975-1977	$^{239}Pu(NO_3)_4$, Inhalation	148
5-1	1965-1967	$^{90}SrCl_2$, Inhalation	63
5-2	1966-1967	$^{144}CeCl_3$, Inhalation	70
5-3	1966-1967	$^{91}YCl_3$, Inhalation	54
5-4	1967-1971	^{144}Ce (FAP)[b], Inhalation	126
5-5	1968-1969	^{137}CsCl, IV injection	66
5-6	1969-1971	^{90}Y (FAP), Inhalation	101
5-7	1970-1971	^{91}Y (FAP), Inhalation	108
5-8	1970-1974	^{90}Sr (FAP), Inhalation	124
5-9	1972-1976	^{144}Ce (FAP), Inhalation (juvenile)	54
5-10	1972-1975	^{144}Ce (FAP), Inhalation (aged)	54
5-11	1972-1975	^{144}Ce (FAP), Inhalation (multiple)	36
5-12	1973-1976	$^{238}PuO_2$, Inhalation (3.0 μm)	84
5-13	1974-1976	$^{238}PuO_2$, Inhalation (1.5 μm)	84
5-14	1977-1979	$^{238}PuO_2$, Inhalation (0.75 μm)	60
5-15	1977-1979	$^{239}PuO_2$, Inhalation (1.5 μm)	108
5-16	1977-1979	$^{239}PuO_2$, Inhalation (3.0 μm)	83
5-17	1977-1978	$^{239}PuO_2$, Inhalation (multiple, 0.75 μm)	72
5-18	1979-1983	$^{239}PuO_2$, Inhalation (juvenile, 1.5 μm)	108
5-19	1979-1982	$^{239}PuO_2$, Inhalation (aged, 1.5 μm)	60
8-3	1967-1973	Gamma ray, whole body, F_3 and F_4 generations	1680
Total	1951-1983		7061

(a) Code designations indicate laboratory and study numbers; 3-5, for example, is a code that indicates laboratory 3 (ANL), study 5. Laboratory codes: 1, U of Utah; 2, UC Davis; 3, ANL; 4, PNL; 5, ITRI; 8 CSU. Study numbers: arbitrarily assigned by NRA.

(b) FAP: radionuclide was adsorbed to an insoluble fused aluminosilicate vector aerosol.

IV: intravenous; SC: subcutaneous

have harvested brain specimens from selected aged dogs to analyze for indicators of Alzheimer's disease, while others have borrowed stomach and mammary carcinoma slides.

Radiobiology Studies

Nearly 40 years ago, the US AEC began life-span, radiation-effect studies in beagles; these closely related experiments are now coming to fruition. The studies, conducted at the University of Utah (U of Utah), UC Davis, Argonne National Laboratory (ANL), Pacific Northwest Laboratory (PNL), and the Inhalation Toxicology Research Institute (ITRI) were summarized by Roy Thompson (1989). His book, *Life-Span Effects of Ionizing Radiation in the Beagle Dog*, became the initial focus of NRA activities. Another multigeneration study in beagles was conducted by the Food and Drug Administration at Colorado State University (CSU). Information from CSU about effects of gamma rays also is being included in the NRA. There also have been many life-span studies of rodents, notably those conducted at Oak Ridge National Laboratory (ORNL), ANL, Brookhaven National Laboratory (BNL), and PNL.

In addition, a series of long-term metabolism studies in nonhuman primates was initiated at the University of Rochester (UR) and continued at Lawrence Berkeley Laboratory (LBL). The most comprehensive description of these studies is given by J. Newell Stannard in *Radioactivity and Health, A History* (Stannard 1988). An excellent summary of the ANL studies was compiled by Doug Grahn in *Studies of Acute and Chronic Radiation Injury at the Biomedical and Medical Research Division, Argonne National Laboratory, 1953-1970: Description of Individual Studies, Data Files, Codes, and Summaries of Significant Findings* (Grahn 1994).

The data from beagle experiments currently available from NRA are listed in Table 1, showing the NRA laboratory-study code, the dates of animal exposure, the nature of exposures (including duration and frequency), and the number of animals held for life-span observation. Table 2 summarizes similar information about rodent and nonhuman primate studies. Information is available on 7061 life-span beagles, 3157 rats, 32,226 mice, and 237 nonhuman primates.

As previously noted, 3 tasks are associated with integrating and preserving information from these studies. The computerized *information system* provides electronic access to summary data on each animal, to document and specimen collection catalogs, and to bibliographic citations about the studies; the *document archives* house and preserve nonbiological materials; and the *specimen archives* house and preserve biological materials.

Advisory Committee

The NRA is guided by the National Radiobiology Archives Advisory Committee (NRAAC), consisting of 5 external advisors: Stephen A. Benjamin, CSU (dog studies); J.A. Louis Dubeau, University of Southern California (molecular biology); Kenneth L. Jackson, University of Washington (radiobiology); Elizabeth E. Sandager, Peabody Museum (archivist); and Philip R. Watson, Oregon State University (databases).

The committee also includes the following 9 participating (or internal) advisors: Bruce B. Boecker, ITRI; Ronald E. Filipy, Washington State University, Tri-Cities; David G. Thomassen, DOE; Bruce A. Carnes, ANL; Scott C. Miller, U of Utah; James F. Park, PNL; Otto G. Raabe, UC Davis; Roy C. Thompson, PNL, and R.J. Michael Fry, ORNL.

Information System

Computer database technology is essential to integrating this broad and diverse collection of information. The NRA is developing 3 interrelated databases, each of which follows the relational model: the dose-effects summary, the collection inventory, and the bibliography. These

Table 2. Major Life-Span Studies (Rodents and Nonhuman Primates) Being Incorporated into the National Radiobiology Archives

NRA Study ID[a]	Dates of Exposures	Description of Study	Number of Life-Span Animals
Rats:			
4-6	1979-1986	Low-level inhaled $^{239}PuO_2$ in Wistar rats	3,157
Total			3,157
Mice:			
7-1	1977	Gamma ray, single exposure at 10 wk, BALB/c & RFM females	4,728
7-2	1987	Gamma ray, single exposure at 10 wk, C3Hf & C57BL/6, both sexes	6,037
7-3	<1979	^{137}Cs, gamma rays, single exposure at 10 wk, RFM, both sexes	19,200
9-1	1982-1987	X or gamma rays, fractionated, various ages, C57BL/6 & CBA/Ca males	3,261
9-2	1985-1989	Low-dose neutron leukemogenesis	-
Total	1977-1989		32,226
Nonhuman Primates:			
6-0		Controls	52
6-1	1954-1982	^{90}Sr	124
6-2	1973-1985	^{239}Pu	28
6-3	1960-1982	^{241}Am	30
6-4	1985-1986	^{237}Np	2
6-5	1976	^{237}Pu	1
Total	1954-1986		237

(a) Laboratory codes: 4, PNL; 6, LBL; 7, ORNL; 9, BNL. Study numbers: arbitrarily assigned by NRA.

systems are on IBM-compatible PC systems at PNL using the Paradox database management system.

Dose-Effects Summary. The computerized summary database contains dose to and effect on each significant tissue in each animal. The summary database has 6 major tables:

LAB:	describing each laboratory
STUDY:	describing each study (as shown in Tables 1 and 2)
GROUP:	describing groups of animals within each study
ANIMAL:	summarizing each animal
TEFFECT:	effect (and diagnosis dates) observed in each significant tissue category
TDOSE:	dose to each significant tissue category at diagnosis dates in TEFFECT.

The summary database also includes laboratory-specific supporting tables for information such as serial hematological determinations or clinical observations. Progress toward populating the summary database is shown in Table 3.

The TEFFECT summary table is based on standardization of clinicians' and pathologists' terminology through SNODOG, an adaptation of the Systematized Nomenclature of Medicine (SNOMED). Last year, 2 documents were published describing the SNODOG glossary and the frequency of usage of its terms in the beagle studies (Watson 1993).

Collection Inventory. The collection inventory database contains information about each bar-code label affixed to materials (or containers of materials) in NRA collections. The database defines materials and tracks location of items for rapid retrieval. More than 15,000 items related to 4500 animals currently are managed by this system.

Bibliography. The bibliographic database uses the collection inventory database's bar-code label system to identify reference materials. Location information about materials is stored in the collection inventory database, and bibliographic citations are stored in the bibliography system. This system includes more than 3000 items of a supporting nature, including animal-specific documents.

An introduction to the NRA information system is available as a stand-alone application that can be self-loaded from diskette onto a DOS-based microcomputer. The documentation accompanying the application, *National Radiobiology Archives Distributed Access User's Manual*, explains usage and extensively describes fields (Watson et al. 1991; Smith et al. 1992). This document and software provide an important summary of the meta-data (information-describing data) collected. The introductory subset diskettes are distributed in response to requests for information about the NRA.

Document Archives

The research document archives contain detailed, "record copy," research findings associated with each study. Materials include handwritten "raw" data such as exposure logbooks, clinical notes, laboratory analysis forms, hematological profiles, and animal-care observations. A significant class of research documents from these studies comprises photographic film, autoradiographs, radiographs, and photographs. "Summarized" data, usually reduced to computer files or publication reprints, also are included. Each document (or document container such as a folder) is given a bar-coded accession-number label and stored in a controlled environment. Material is catalogued in the bibliographic database for rapid selection and retrieval.

The first contribution to the document archives is the extensive collection of supportive documentation that provided the basis for *Radioactivity and Health: A History* (Stannard 1988); 63 boxes have been accessioned (Watson et al. 1994). In addition, 39 lineal feet of clinical records and an extensive collection of radiographic records were transferred from UC Davis in June 1992. In 1990, documents from the U of Utah such as clinical records, radiographs, photographs, and autoradiography preparations, as well as specimens such as organs, histology blocks, and slides, were accessioned; these materials will remain in Utah pending completion of the studies.

Specimen Archives

The biological specimen archives contain collected research materials such as tissues preserved in formalin or alcohol, tissue samples embedded in paraffin or plastic for histopathological analysis, microscope slides, and radiographic films. Many materials are radioactive and associated with hazardous materials such as formalin, alcohol, or paraffin. A building has been renovated to serve as the repository of these specimens. The building contains a specimen-manipulation laboratory, storage bays, and an automatic fire-suppression system. Materials are nominated for donation to the NRA by an institution which recognizes that specific completed studies are worthy of consideration for archival preservation.

Collaborations and Retrievals

Cooperation of participating institutions and investigators is essential to achieve goals of the NRA project. Collaboration has been excellent with the 9 institutions that have donated

Table 3. Progress Toward Populating the Summary Database

NRA Lab and Study ID[a]	Status of NRA Database Tables[b]						
	LAB	STUDY	GROUP	ANIMAL	TEFFECT	TDOSE	LAB SPECIFIC
1-1 to 1-14	F	C	C	C	C	C	C
2-1	F	C	C	P			
2-2 to 2-04	F	C	C	C	C	C	C
3-1 to 3-03	F	C	C	P			
3-4	F	C	C	C	C		C
3-5, 3-06	F	C	C	P			
4-1, 4-02	F	C	C	P		P	P
4-3 to 4-05	F	C	C	C	P	P	P
5-1 to 5-19	F	C	C	C			
6-1 to 6-3	F	C	C				
6-4 to 6-5	F	C	P				
7-1, 7-2	F	C	C	C	C	C	C
7-3	F	C	C	I			
8-3	F	P	P	I	I		
9-1	F	C	C	P			
9-2	F	C					
10-1 to 10-51	I	I	(Detailed information was *discarded* by University of Rochester; NRA has reprints of results and study definition records only.)				
Number of Records:	9	126	545	19,883	63,318	7,911	>250,000

(a) Laboratory Codes: 1, U of Utah; 2, UC Davis; 3, ANL; 4, PNL; 5, ITRI; 6, LBL; 7, ORNL; 8, CSU; 9, BNL; 10, University of Rochester. Study numbers are defined in Tables 1 and 2.

(b) Status Codes:
C, Complete: database records are complete; all significant fields have complete information.
F, Final: database records are complete and reviewed by investigator.
I, Incomplete: database tables are partially filled with representative rows.
P, Partial: database records are partially complete; some fields have no information.

information and materials. NRA staff have participated in, or have been invited to participate in, several site visits; collaborative projects were initiated, and these laboratory directors serve on the NRAAC.

The NRA encourages analysis of studies that examine previous information from a new perspective by applying different analytical approaches, or by comparing results of studies performed at different institutions. The NRA collaborated with investigators at UC Davis to obtain brain specimens of dogs whose clinical records indicated Alzheimer-like symptoms. NRA staff retrieved tissues and provided laboratory facilities, and the UC Davis team prepared histopathology slides for staining and interpretation (Russell et al. 1992). In addition, the NRA specimen archives supplied histopathology slides of control-beagle stomach tissue to the veterinary school at UC Davis and brain specimens to the University of Tennessee. The information system responded to several requests for detailed data subsets.

A subcommittee of the NRAAC met in December 1991 to plan a collaborative database combining information from 1096 control beagles. The NRA is coordinating publication of this reference set to provide baseline information for comparison with experimental groups. A

consortium of biostatisticians from ANL, ITRI, and PNL will analyze this control beagle subset.

Collaboration with the Europeans

A similar archiving task has been initiated by the Commission of European Communities (CEC). The ERA is being developed for the European Late Effects Program (EULEP) by Dr. Georg Gerber. The NRA is actively cooperating with Dr. Gerber to coordinate database design, with the goal of eventual integration. Agreement on computer hardware and database management software was reached, and the database structures are being merged. A joint publication of descriptive information is planned for spring of 1995.

Future Activities

The NRA will continue the orderly accessioning of life-span beagle-study information, and shipment of selected specimens and documents to PNL. While these studies are being completed, NRA will play an increasing role in facilitating analyses that cut across studies and species. For example, NRA will compile and publish a combined data set of control beagles. Because most rodent-based radiobiology studies involved thousands of animals, access to original, unpublished data from them is limited. Therefore, the NRA will continue to solicit details about additional rodent studies, initially those conducted at ANL, BNL, ORNL, and PNL. The NRA will work closely with the interlaboratory consortium of statisticians developing techniques for comparing and combining information from the beagle studies.

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Statistical Analyses of Data from Radiobiologic Animal Studies

Principal Investigator: E.S. Gilbert

Other Investigators: R.L. Buschbom, R.L. Hill

The purpose of this project is to enhance the information extracted from life-span studies in experimental animals, especially those conducted in beagles, by providing statistical methods and analyses that are as unbiased and informative as possible for assessing human health risks. Statisticians from Pacific Northwest Laboratory (PNL), Argonne National Laboratory (ANL), and the Inhalation Toxicology Research Institute (ITRI) are applying state-of-the-art statistical methods to past and current data generated by scientists involved in the U.S. Department of Energy (DOE) experimental radiobiology program. The project also involves developing statistical methods and software to meet special challenges posed by these radiobiology studies, and improving methods for estimating organ-specific doses from internally deposited radionuclides. A major objective is to establish an integrated approach for synthesizing data across DOE laboratories. This approach includes both standardized analyses, in which estimates of risk comparable across studies and across exposure groups will be obtained for all studies meeting specified criteria, and more extensive and specialized analyses based on combined data from selected studies. In combination, the standardized and combined analyses are expected to provide the best possible overall assessment of DOE radiobiologic data, and to allow these data to be used in an optimal way for assessing human risks.

Introduction

A wide variety of radiobiologic animal experiments, primarily in rodents and beagles, have been conducted for the DOE at several laboratories. Most of the DOE radiobiologic studies in laboratory animals were designed to obtain information for exposure situations in which adequate human data are unavailable, and particularly to provide information on how dose, dose rate, dose distribution, LET, and other factors modify risks. Radiation exposures from both external sources and internal emitters have been studied. In this project, we hope to increase the understanding of radiation-related human health risks by developing and implementing a plan for analyzing and integrating the extensive data that have been generated from DOE life-span animal experimental studies. To extend statistical analyses now being conducted at individual laboratories, project researchers apply common approaches to data from different studies, conduct analyses that directly combine and compare data from related studies, and further develop the statistical methods and computer software needed to meet special challenges posed by these data. The laboratory animal data from DOE radiobiologic experiments represent an extremely important resource of information on radiation exposures for which human data are unavailable, and the recent development of powerful statistical tools and software for analyzing survival data now makes it feasible to provide a more rigorous and informative assessment of these data than has been possible in the past.

The objectives of statistical analyses conducted under this project are to provide measures of carcinogenic risk that can be appropriately compared across studies, to quantify differences in risks produced by various radionuclides and exposure conditions, to compare risks across species, to investigate the shapes of dose-response functions in a consistent manner, and to evaluate the consistency of results with models that have been used for human radiation risk assessment. These analyses will provide information needed for estimating risks in man, and will thus decrease the uncertainty in risk estimates, especially for exposure situations not represented by human data.

Approach

Statisticians and experimenters from PNL, ITRI, and ANL are collaborating to develop an integrated approach to analyze data from past and present DOE radiobiological studies, especially those conducted in beagles. The project includes regular meetings of the 3 principal investigators (statisticians from PNL, ITRI, and ANL), with participation of radiobiologists, dosimetrists, pathologists, representatives from the National Radiological Archives (NRA), and others who are involved in the studies. The project includes both standardized and combined analyses, as described below.

Standardized analyses of each experiment include calculating a measure of lifetime risk and its uncertainty using specifically recommended procedures for each group of animals defined by dose level, type of radiation exposure, and possibly other factors. These analyses also include the fitting of specified dose-response functions. Such standardized analyses do not preclude other types of analyses that might be appropriate for particular studies, but allow use of these data for human risk assessment in ways that may not be currently anticipated.

In addition to the standardized analyses, we are working with the investigators who conducted the experiments to determine areas in which further *combined analyses* are needed. These more detailed analyses combine data from selected radiobiologic studies to address specific questions of interest, and they give careful consideration to comparability of (1) dose estimates and their uncertainties; (2) data on health endpoints, including pathologists' judgments in identifying types of tumors and assigning cause of death; and (3) baseline risks. Used carefully, analyses based on combined data permit the development of combined risk estimates, and provide an assessment of differences in risks from various types of exposure that is more rigorous than could be obtained if only analyses based on data from individual studies were available.

Progress in FY 1994

This summary focuses on activities in which PNL researchers participated, and excludes activities primarily conducted by ITRI or ANL researchers. Three meetings of the principal investigators were held (December 1993 at ITRI, April 1994 at ANL, and November 1994 at PNL), and the following tasks were accomplished.

Draft protocols for standardized analyses and for statistical methods have been prepared. The protocol for standardized analyses discusses factors such as: statistical methods and models; how dose groups are to be defined; treatment of variables such as sex; health endpoints that will be evaluated; dosimetry; context of observation, including the use of radiographic data; baseline risks; and measures to be used to summarize data. Because many statistical methods used to analyze experimental data are common to both standardized and combined analyses, a separate document on statistical methods has been prepared. This document describes models for the hazard or age-specific risk, and gives special attention to appropriately accounting for the context in which tumors are observed (e.g., fatal or incidental to the death of the animal).

We have begun using analyses of control dogs as the first example of combined analyses, and to test the use of the NRA for obtaining data. These analyses will bring together in one place descriptive information on the control populations, including simple information on the number of animals, number of tumors of various types, average life span, etc. The analyses also will include a rigorous statistical assessment of differences in several health endpoints by laboratory, sex, and individual studies within laboratories. Based on these results, it may be possible to identify groups of control animals that can be appropriately used for a variety of specific purposes.

Initial analyses of data on rats exposed to inhaled $^{239}PuO_2$ in studies at PNL and ITRI have been conducted. Further work addressing

comparability of both pathology and dosimetry in the 2 studies is underway, and will be followed by additional analyses. To implement the effort conducted at PNL, we developed software for conducting analyses that account for information provided by the pathologist on whether tumors were fatal or incidental to the death of the animal. The software is sufficiently flexible to (1) fit a variety of dose-response functions, such as linear-quadratic and power functions; (2) quantify estimates of both the hazard and lifetime risks; (3) assess the dependence of risks on dose rate, type of exposure, and other factors; and (4) investigate changes in the hazard as animals are followed over time. Initial documentation of this software has been prepared and provided to statisticians at ANL and ITRI.

Analyses of rats exposed to radon at PNL also have been conducted, and a draft paper describing these analyses prepared. Although these data have been analyzed previously (Gilbert 1989), additional data on studies of low exposures and exposure rates have since become available. Moreover, earlier analyses did not utilize pathologists' judgments on whether tumors were fatal or incidental. Combined analyses of epidemiologic data from 11 cohorts of underground miners recently have been published (Lubin et al. 1994), and have provided new models, particularly for evaluating the effect of dose rate on risks. It is of interest to test these models in experimental animals.

Efforts have begun in dosimetry, an important component of this project, particularly for animals exposed to inhaled radionuclides. Initial work in this area involves dogs exposed to inhaled $^{238}PuO_2$ at PNL and ITRI, with particular attention to ensure that methods being used to estimate organ doses in this study are comparable for the 2 laboratories. These efforts will be extended to estimate lung dose in rats exposed to particles of $^{239}PuO_2$ at PNL and ITRI.

To estimate the temporal accumulation of dose in inhalation studies, work has been initiated to evaluate several quantities: (1) initial lung deposition (ILD), which is the initial amount of material deposited at each exposure in the deeplung, not including rapidly cleared material in upper airways; (2) tissue weight, ideally temporally based to allow for growth of the animal after exposure; (3) parameters describing the radioisotope's temporal distribution in the tissues for which dosimetry estimates are desired; and (4) the fraction of the energy absorbed in the target tissue. Data available for estimating these quantities include information on the tissue-specific distribution of material in each animal at the time of death, and, in some studies, one-time or periodic whole body counts, excreta data, or weights (usually body weight and weights of selected tissues at necropsy).

The Future

Standardized analyses of data from all DOE life-span animal studies meeting specified criteria will be completed. The results of these analyses and a description of the methods used to obtain them will be published in a summary report, and will be submitted in an abbreviated form to a peer-reviewed journal. Comparable summary statistics and estimates of their uncertainty will be provided for each group of animals within each qualifying study. These statistics will then be available for use by those interested in radiation risk assessment, and by those interested in a general understanding of the radiation-carcinogenesis process. The summary measures could be used in analyses ("meta-analyses") to address questions that may arise in the future.

Further combined analyses of data from selected studies will be conducted, and will address certain issues in more detail than the standardized analyses. These analyses will give careful attention to comparability of dosimetry, pathology, and other aspects of the experimental protocol. Methodological problems posed by these analyses will be explored, and adequate statistical approaches for addressing them will be developed.

In combination, the standardized analyses and the combined analyses are expected to provide the best possible overall assessment of DOE experimental data, and to allow these data to be used in an optimal way for assessing human risks.

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Genotoxicity of Inhaled Energy Effluents

Principal Investigator: A.L. Brooks

Other Investigators: K.M. Groch, B. Wood, and R.F. Jostes

This project focuses on 2 major areas of research. First, we are evaluating the use of cellular and molecular techniques to help understand risks associated with inhalation of complex mixtures. These studies required development of a model system to expose cells directly to the vapor-phase materials in complex mixtures. Second, studies are being conducted to evaluate chromosome aberrations as an indicator of early change in carcinogenesis. Previous mechanistic studies employed human chromosome probes in A_L cells to compare dose-response relationships for the induction of chromosome aberrations (measured as color switches) following high-LET (radon) and low-LET (^{60}Co) irradiation. We determined that the color switches are adequate to describe these dose-response relationships. In a continuation of the mechanistic studies associated with chromosome aberrations, we have used probes that "paint" the chromosomes of rat cells via fluorescent in situ hybridization (FISH). Dose-response curves have been developed for low-LET irradiation in primary rat-skin fibroblasts using chromosome 1 probes. In other experiments with X and Y probes in the rat, it was demonstrated that it is possible to determine the sex of rodent cells during the interphase stage of the cell cycle. This research was very useful in leukemia transplant studies conducted in rats, and could have application in other studies that require the evaluation of the sex of interphase cells.

Methods

Model Exposure Systems for Airway Epithelium

We have developed an exposure system with a controlled rocker arm to irradiate cells of the airway epithelium. This rocker arm allows cells in half the flask to be covered with medium at all times while cells in the other half of the flask are exposed directly to vapor-phase materials. The system makes it possible to conduct studies on cell survival and on genotoxicity as measured by induced chromosome aberrations, micronuclei, sister chromatid exchanges (SCEs), and mutations. The Pacific Northwest Laboratory (PNL) is currently working with Westinghouse Hanford Company to evaluate the biological activity of vapor-phase samples from waste tanks with this system.

Mechanisms Involved in Chromosome-Aberration Production

A_L cells were exposed to ^{60}Co at a dose rate of 0.5 Gy/min and total doses of 0.0, 1.0, 4.0, 8.0, and 10.0 Gy to generate dose-response relationships for color switches (a measure of induced aberrations) between human and rodent chromosomes. Additional cells were exposed to 0.0 or 1.2 Gy from radon using the in vitro exposure system described by Jostes et al. (1991). Radon-exposed cells, and cells exposed to 4.0 Gy of ^{60}Co gamma rays, were harvested at 1, 3, 9, and 15 days after exposure. During the time between exposure and harvest, cells were maintained in exponential growth by subculturing every 4 days. The chromosomes were painted using the technique published by Pinkel et al. (1986), and the number of color junctions were scored. Color junctions are defined as the interface between a yellow-stained human chromosome 11 and a red-stained propidium iodide-labeled Chinese hamster ovary (CHO) chromosome. Aberrations in other chromosomes were not recorded.

Molecular probes were developed in collaboration with Lawrence Livermore National Laboratory (LLNL), Livermore, California, to paint selected rat chromosomes. These probes were designed, using in situ methods previously developed for mouse chromosomes (Breneman et al. 1993), to label enough rat genome to determine dose and induced chromosome translocations during tumor development, and to identify

X and Y chromosomes for gender identification of rat cells in tumors.

Interphase cells from the spleen of healthy male and female mice, and from the spleen of female mice that had leukemia induced by injection with male leukemia cells, were evaluated for copies of X chromosome. These studies tested the hypotheses that the injected cells undergo clonal expansion and are of male origin. The dose-response relationships for the induction of chromosome aberrations were evaluated using fluorescent in situ hybridization (FISH) in primary cultures of skin fibroblasts established from female Fischer rats (55 to 60 days old) in Dulbeco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 15 m*M* HEPES (pH 7.4), and 50 μg/ml gentamycin. Briefly, the abdomen of an animal was shaved and washed with iodine, then several 5x5-mm pieces of skin were excised. Three pieces of skin were placed in a 100-mm tissue-culture dish and covered with a sterile glass cover slip. The cover slip was attached to the dish with a small amount of sterile silicon grease. The tissue was incubated at 37°C with 5% CO_2 until the dish was ~75% confluent. The cover slip then was removed and the cells obtained from both surfaces with trypsin. The cells were passed at about 75% confluency and a dilution of ~1:10 into secondary culture using the same medium. After three passes, cells were resuspended in the growth medium described previously, but further supplemented with 15% dimethyl sulfoxide; aliquots then were cryopreserved in liquid N_2. Ninety-six hours before exposure, an ampoule of cryopreserved cells was thawed and cultures were reestablished using the growth medium described previously.

Cell densities were adjusted so that cultures were approximately one-third confluent at the time of irradiation. Twenty-four hours before exposure, the culture medium was replaced with fresh growth medium. The exponentially growing cultures were irradiated in the ^{60}Co gamma cell facility at PNL at an exposure rate of ~1 Gy/min and doses of 0.0, 1.0, 2.0, and 3.0 Gy. Following exposure, the cultures were returned to the incubator for 18 hours before addition of 70 ng/ml colcemid; the cultures then were incubated with colcemid for an additional 4 hours. The culture medium containing nonadherent cells was decanted and the adherent cells removed from the dishes with trypsin. The adherent and nonadherent cells were pooled, their suspension adjusted to 10% fetal bovine serum, and pelleted. The cells then were washed in Hank's Balanced Salt Solution (HBSS), pelleted, and resuspended in hypotonic 75 m*M* KCl for 10 minutes, fixed in 3 changes of ice-cold methanol/glacial acetic acid (3/1), resuspended in methanol/acetic acid, and dropped onto cold, wet slides. The slides were allowed to cure for 48 hours at room temperature, then stored at -20°C under N_2 until staining.

Slides for staining were denatured in 70% formamide containing 2x SSC (1x SSC = 0.15 *M* NaCl, 0.015 *M* $Na_3C_6H_5O_7$), at a pH of 7.0, for 5 minutes at 70°C. The slides were then dehydrated in an ascending series of ethanols at -20°C and dried under a jet of N_2, then placed on a warmer at 35°C. A biotinylated DNA probe for chromosome 1 was denatured in 70% formamide containing 2x SSC at pH 7.0, 0.5 ng/ml dextran sulfate, and 0.1 ng/ml sonicated salmon sperm DNA for 5 minutes at 70°C. Thirty-five μL of denatured probe was applied to each slide; a 22x50-mm cover slip was applied to the slide and sealed with rubber cement. Hybridization was allowed to proceed at 35°C overnight.

Following hybridization, slides were washed 5 times: 3 times with 50% formamide containing 2x SSC at pH 7.0, 1 time in 2x SSC at pH 7.0, and 1 time in 2x SSC at pH 7.0 containing 0.05% NP-40. All post-hybridization washes were at 41°C. Chromosomes with bound probe were visualized by treating with fluoresceinated avidin (5 μg/ml) followed by an amplification step of biotinylated mouse anti-avidin antibody (5 μg/ml). After a second application of fluoresceinated avidin, the stained slides were washed in distilled water and dried. Metaphases were counterstained with 0.3 μg/ml propidium iodide in 75% glycerol containing 9 mg/ml *O*-phenylenediamine·2HCl (anti-fade).

Metaphase spreads were visualized with epifluorescent illumination on a Nikon Optiphot equipped with a 100-watt Hg lamp and appropriate excitation/barrier filters. Spreads were analyzed in groups of 100, and photographs of

any abnormalities were made with 200 ASA Ektachrome film. Potential aberrations evident in the photographs were scored by at least two independent observers without prior knowledge of the exposure conditions.

Probes for both chromosome 1 and 2 were evaluated for potential use. Because differential staining was best for chromosome 1, cytogenetic evaluations of damage were limited to this chromosome.

Results and Discussion

Model Exposure Systems for Airway Epithelium

Preliminary experiments using this exposure system focused on cell killing and the induction of SCEs in CHO cells. The system can be calibrated to measure a range of cell killing by vapor-phase materials. The frequency of induced SCEs can be measured at levels of exposure where cell killing is minimal.

Mechanisms Involved in Chromosome-Aberration Production

The goal of the first study employing rat chromosome probes was to assess whether it was possible to determine the sex of leukemia cells in a rat large granulocytic leukemia (LGL) model. Figure 1 illustrates the number of fluorescent labeled spots that represent the X chromosome in interphase cells from male and female rats. A certain fraction (10 to 20%) of the interphase cells do not seem to take up stain on the X chromosome. In males, most of the cells have either no label or a single label; very few cells have two bright spots. Most cells from spleens of female rats have two labels. Some cells in the female show only a single bright spot while others have no label. This phenomenon seems to be related to the way the cells are oriented on the slide; when both bright spots are in close enough proximity, they are scored as a single spot. A female rat that has LGL induced by injection of male rat cells is shown in Figure 2. There is a single bright spot (male cells) in most cells; only one cell in this field has two bright spots (female cell). This finding shows that the cells responsible for the tumor are of male origin. As previously postulated, after injection cells

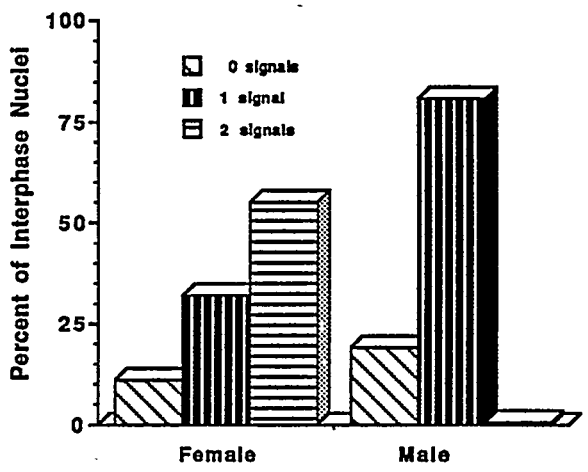

Figure 1. The Frequency of X Chromosomes in Interphase Cells Labeled With Whole Rat Chromosome Probes. The male cells have a single copy of the X chromosome; the female cells appear to have 1 or 2 copies.

underwent clonal expansion and did not transfer the leukemic phenotype to female cells.

The frequency of color junctions in A_L cells was evaluated as a function of dose, time after exposure, and LET. The findings indicated that the increase in color junctions was a nonlinear function of radiation dose (Figure 3), which was described by the following linear-quadratic function: color junctions/cell = $0.027 - 0.011 D + 0.0097D^2$ ($R^2 = 0.99$). The time course for elimination of cells with color junctions from the population is shown in Figure 4. The graph illustrates that after the initial clearance of cells with damage by ^{60}Co, there was a rather constant level of chromosome damage. For radon-exposed cells, the level of color junctions remained rather constant. The relative biological effectiveness of radon relative to ^{60}Co in this study was 3.6 when the response was compared at a constant dose (1.2 Gy) and time (24 hours).

The dose-response relationship for the induction of color junctions was evaluated in rat fibroblasts using a chromosome 1 paint. A breakdown of the types of aberrations scored is shown in Figure 5. It was noted that most of the

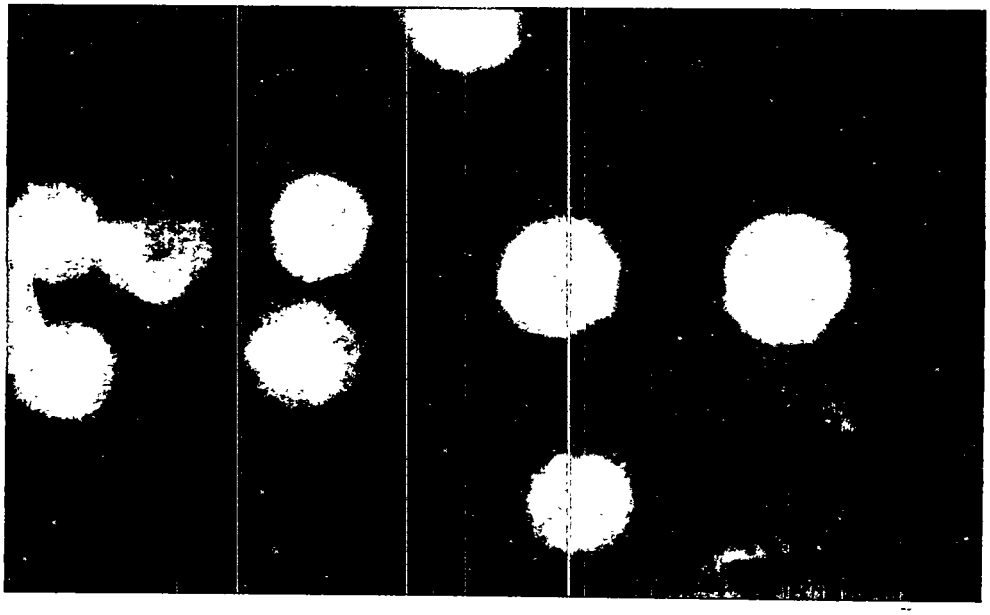

Figure 2. Spleen Cells From a Female Rat Infected With Large Granulytic Leukemia Cells From a Male Rat. The single X chromosome in the interphase cells demonstrates that the leukemia cells are of male origin.

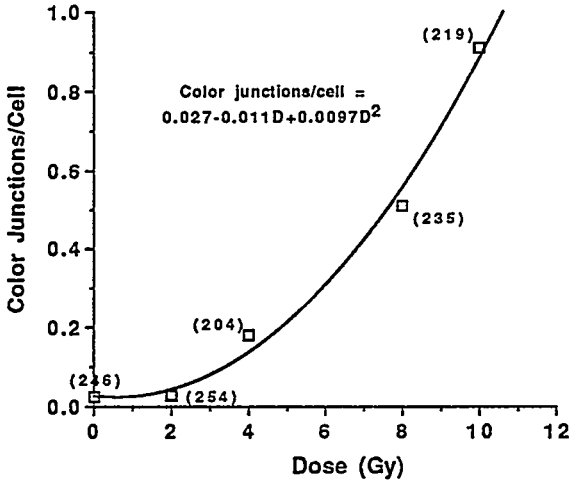

Figure 3. The Dose-Response Relationship for Induction of Color Junctions in A_L Cells by ^{60}Co Exposure

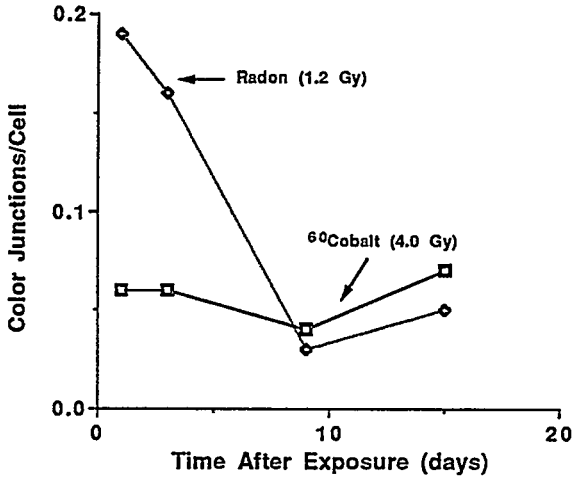

Figure 4. The Time-Response for Survival of Color Junctions in Dividing A_L Cell Populations in Culture Following Either 4.0 Gy of ^{60}Co Gamma Radiation or 1.2 Gy of Alpha Particles from a Radon Source

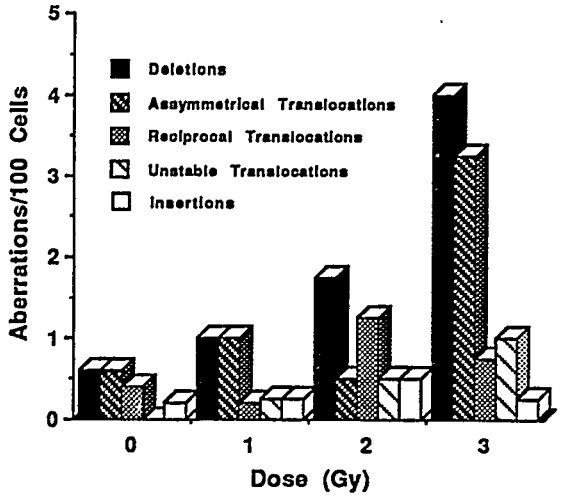

Figure 5. The Distribution of Aberration Types in Primary Rat Skin Fibroblasts Measured by Color Junctions 18 Hours After Exposure to ^{60}Co Gamma Radiation

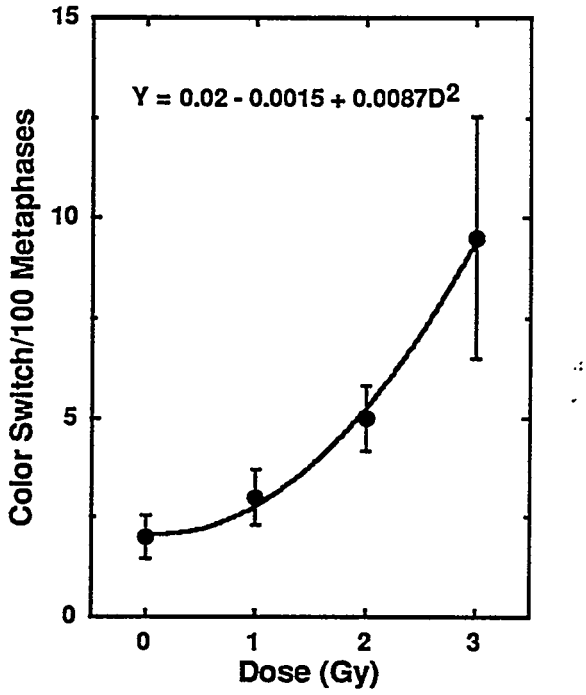

Figure 6. The Dose-Response Relationship for the Induction of Color Junctions in Primary Rat Skin Fibroblasts Exposed to ^{60}Co Gamma Rays. The data is for changes that involve rat chromosome 1.

aberrations scored at 18 hours after exposure were of the chromosome type. The data also suggest that the frequency of asymmetrical translocations was similar to that observed for deletions and was higher than for reciprocal translocations or unstable chromosome translocations (rings + dicentrics). The frequency of reciprocal translocations was not different from that seen for unstable chromosome translocations. The dose-response relationship for total color junctions is illustrated in Figure 6, which illustrates that the frequency of aberrations again increases as a nonlinear function of radiation dose according to the equation color junctions/cell = $0.02 - 0.0015\ D + 0.0087D^2$ ($R^2 = 0.99$). This is not significantly different from the dose-response relationships for the induction of color junctions in A_L cells. Color junctions can be used to evaluate dose-response relationships for rodent cells even when there are no probes available to identify centromeres.

These chromosome probe methods will be employed in future studies of induced aberrations in cells following exposure to combinations of well-defined vapor-phase mixtures and ionizing radiation.

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Molecular Events During Tumor Initiation

Principal Investigator: D.L. Springer

Other Investigators: A.O. Murad and D.B. Mann

The primary objective of this project is to test the hypothesis that chromatin structure modulates chemical-induced carcinogenesis. Recently, we demonstrated that benzo(a)pyrenediol epoxide (BPDE)-induced DNA damage is modulated within nucleosomal DNA. Currently, we are investigating formation of BPDE adducts and their removal from a specific sequence with a well-defined chromatin structure and transcriptional regulation. For this purpose we are using the mouse cell line L1.4-3, with a stably integrated construct consisting of the herpes simplex virus thymidine kinase (*tk*) gene linked to the mouse mammary tumor virus (MMTV) long terminal repeats (LTR). (This construct is designated LTL.) The *tk* gene is transcribed from the glucocorticoid-inducible promoter located in the upstream LTR. We have quantitated overall adduct levels and their removal from the genomic DNA in intact cells and in the LTL sequence (as naked DNA) using radiolabeled BPDE and quantitative polymerase chain reaction, respectively. We find that about 50% of the adducts are removed from genomic DNA in 24 hours. In comparison, indirect measurements using northern blot analysis of the *tk* gene indicate that transcription from this sequence is inhibited by >70% when measured 1 hour after treatment with 4.8 μM BPDE and recovers to >60% of control levels (untreated cells) in 24 hours. This inhibition follows single-hit kinetics, suggesting that BPDE adducts to DNA template are directly responsible for this effect.

Introduction

Benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), the ultimate carcinogenic metabolite of benzo[a]pyrene, covalently binds to DNA predominantly at guanine residues. The primary target of covalent modification is through trans addition of (+)-anti-BPDE to the exocyclic amino group of guanines. Covalent adduct formation by BPDE interferes with a number of cellular processes, including DNA replication and transcription, and is thought to be a critical event in tumor initiation. Because most DNA in eukaryotic cells is closely associated with histones, an accurate understanding of carcinogen binding to DNA must consider the role of chromatin structure. Previously, our research was centered around the influence of nucleosome structure on the location and extent of DNA damage. Results from this work demonstrated that the DNA was partially protected from BPDE damage near the center of the nucleosome, and that the rotational setting of the guanines on the helix did not influence carcinogen binding. We now are conducting experiments to investigate the dynamics of adduct formation and the efficiency of their removal from specific genomic sequences for which precise chromatin structure and interaction with transcription factors are known. We are looking at how adduct formation in specific regions of this sequence affects transcription from this sequence, and the time course and mode of removal from these specific regions. For this purpose we have obtained a mouse cell line containing a stably integrated construct derived from mouse mammary tumor virus (MMTV; Zaret and Yamamoto 1984). This construct consists of the MMTV provirus from which most of the viral sequence between the 2 long terminal repeats (LTR) is deleted and replaced by the thymidine kinase (*tk*) gene from the herpes simplex virus. (The construct is designated LTL; Figure 1.) The mouse L1.4-3

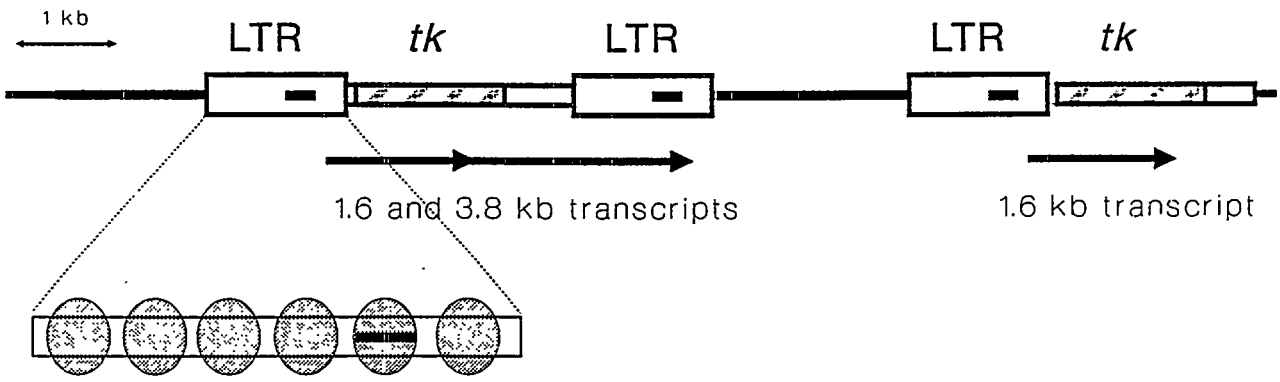

Figure 1. Schematic of the LTL Construct in L1.4-3 Cells. Two transcripts produced from this construct also are shown. The major transcript is the 1.6 kb; the 3.8-kb transcript is a result of a leaky poly A site in the *tk* gene. A phased array of 6 nucleosomes is apparent on each of the LTR (Richard-Foy and Hager 1987; Beato et al. 1991).

cell line contains a single 1½ tandem copy of this sequence stably integrated into its genome. The *tk* gene in this construct lacks its own promoter and is transcribed from the glucocorticoid hormone-inducible promoter located in the upstream LTR. Hormone inducibility of transcription from MMTV-LTRs is orchestrated by the precise positioning of 6 nucleosomes (Figure 1) and ensuing changes in chromatin structure in the cells. Structural changes that occur in this sequence after binding of hormone-receptor complex to its cognate sequence have been extensively investigated and reported. It has been shown that one of these nucleosomes is disrupted upon binding the hormone receptor complex. Treatment of cells with the synthetic glucocorticoid hormone, dexamethasone, results in the appearance of a broad DNase-I hypersensitivity and specific DNase-I hypersensitive sites (Zaret and Yamamoto 1984). These results indicate that the chromatin structure actively participates in regulation of transcription. Moreover, features of the chromatin structure that regulate the binding of transcription factors and poise chromatin for different cellular functions may also influence DNA damage and repair processes (Smerdon 1991). While it is known that repair of many genes is coupled to transcription (Selby and Sancar 1994; Smith 1987), recent studies with ultraviolet (UV) photoproducts indicate that the structure the DNA adopts in cells not only influences the damage but also the repair at specific sites and regions (Gao et al. 1994; Lobanenkov et al. 1986; Tornaletti and Pfeifer 1994). This site- and region-specific repair of damage may not be passive; it may play an important role in maintaining genomic integrity under adverse conditions and, thus, survivability of a cell. Many oncogenes undergo site-specific mutations in order to be activated. The MMTV-LTR system provides a unique opportunity to investigate these aspects of DNA damage and repair in the hope of ascertaining the underlying principles governing the mechanism that controls these processes.

Methods/Results

We determined a useful dose range for BPDE treatment of L1.4-3 cells and the efficiency with which these cells remove resulting adducts from genomic DNA. Cells were incubated with varying concentrations of [^{3}H]-(±)-anti-BPDE in growth medium. After 1 hour of incubation, cells were harvested, lysed in a buffer containing

sodium dodecyl sulfate (SDS) and treated with proteinase K. DNA was purified by 2 successive ethanol precipitations, and the amount of radioactivity in 50 μg of purified DNA from each of the samples was determined by liquid scintillation counting. Exposure of these cells at concentrations from 0 to 10 μM (±)-anti-BPDE produced a linear dose-response curve with a slope of 0.07 adducts/kb/μM BPDE (Figure 2A). To determine repair efficiency, cells were treated in a similar manner at BPDE concentrations of 2 and 4 μM, but they were allowed to repair for various times up to 24 hours in the presence of 2 mM hydroxyurea to suppress replication. We estimate that >95% of these cells were able to survive 4 μM BPDE treatment in this quiescent state. As can be seen in Figure 2B, at least 50% of adducts were removed by 24 hours from these cells, in contrast to the repair of UV photoproducts, where only 15 to 20% were removed from bulk chromatin in 24 hours, as reported by others for rodent cell lines (Smith 1987).

It is important to determine how BPDE adducts that are formed in specific sequences affect the initiation (induction) and elongation of transcription in that sequence. The MMTV model presents an ideal system by providing an "on/off" switch to control transcription externally by addition and removal of glucocorticoid hormone. In the absence of hormone there is a very low level of constitutive transcription (not enough for *tk* cells to survive in medium containing hypoxanthine, aminopterin, and thymidine), which increases more than 50-fold upon addition of the synthetic glucocorticoid hormone, dexamethasone. Moreover, since *tk* mRNA belongs to the short-lived class of RNA, with a half-life of only 55 minutes, northern blot analysis of *tk* mRNA can be used to directly analyze rates of transcription from this sequence. Figure 3 contains the data for one such experiment, in which cells were incubated with various concentrations of (+)-anti-BPDE for 1 hour; then *tk* mRNA transcription was induced by growing cells in the presence of dexamethasone for an additional 1-hour period. At this time cells were immediately lysed in a guanidine thiocyanate lysis buffer, and total cellular RNA was isolated and analyzed by northern blot analysis. The linearity of dose-dependent

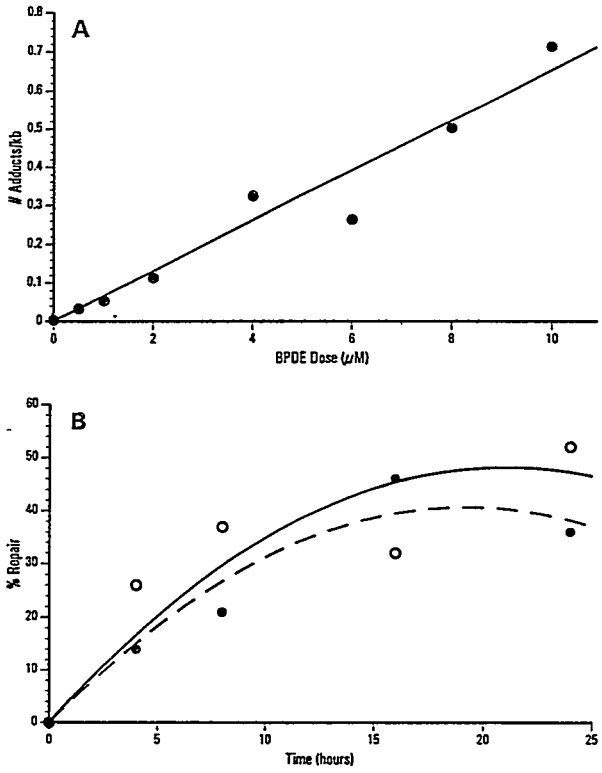

Figure 2. Analysis of Benzo(a)Pyrenediol Epoxide (BPDE) Adducts in Genomic DNA and Overall Rate of Repair. (A) Dose-response curve obtained by incubating cells with varying concentrations of [^{3}H]-labeled (±)-anti-BPDE. Amount of radioactivity incorporated in DNA was used to calculate adduct levels in genomic DNA. (B) Time course for removal of these adducts from bulk genomic DNA of cells. Cells were incubated in 2 mM hydroxyurea to block replication during repair incubation.

inhibition of *tk* mRNA on a log scale is indicative of single-hit kinetics. This suggests that inhibition of transcription of the *tk* gene is a direct result of adducts formed in the sequence required for transcription and is not due to secondary effects, such as damage to proteins or other factors required for transcription.

When the cells were allowed to repair in a quiescent state, the *tk* mRNA levels returned to near control level (unadducted) within 24 hours, indicating removal of adducts from the DNA template (Figure 4). This recovery appears to be more rapid than repair to the bulk DNA (see Figure 2B), suggesting that the transcriptionally active *tk* gene may be repaired preferentially (as

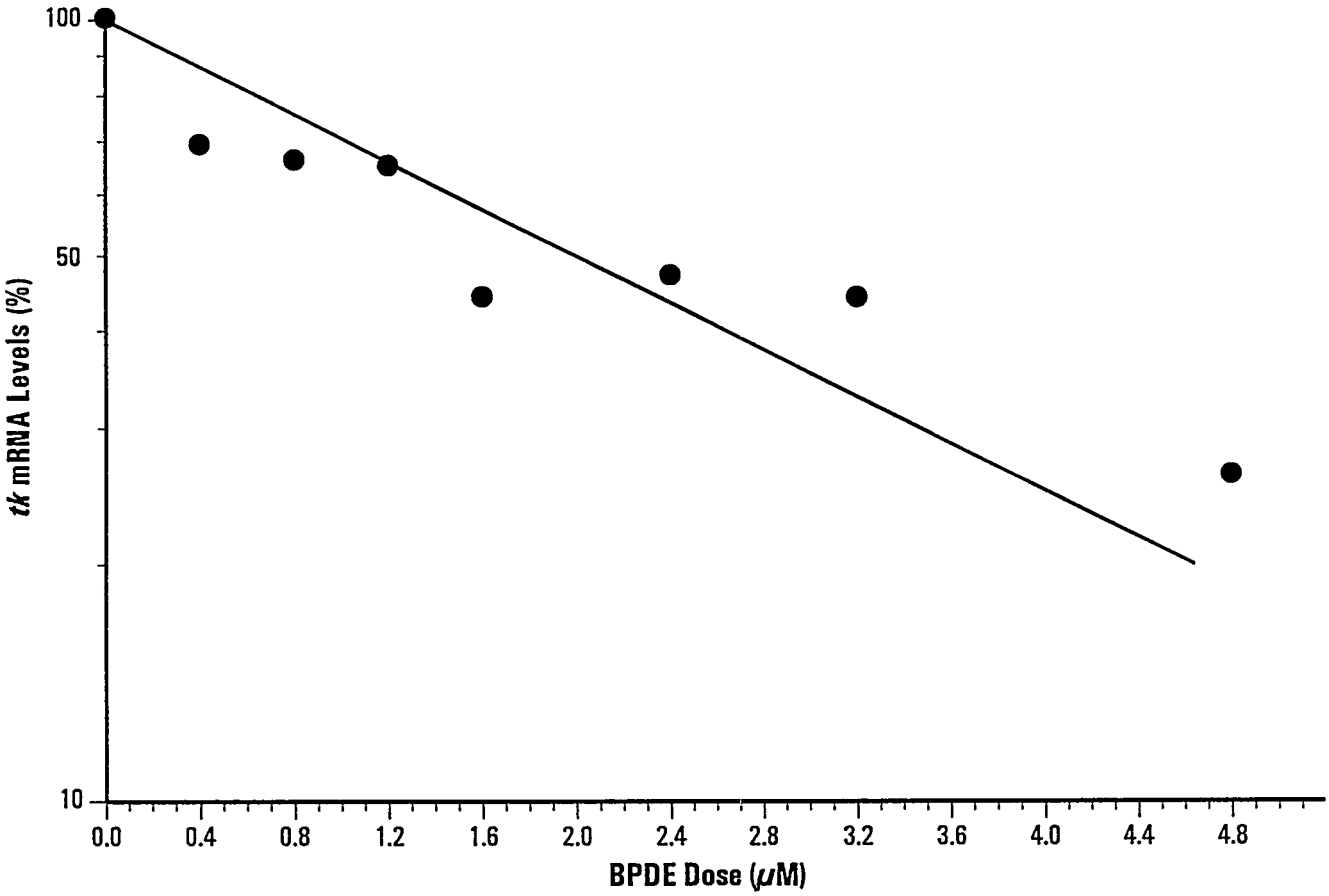

Figure 3. Inhibition of *tk* Gene Transcription with BPDE Treatment. L1.4-3 cells were incubated with 0-4.8 μM (+)-anti-BPDE for 1 hour. Cells then were washed with phosphate-buffered saline and incubated in growth medium containing 0.1 μM dexamethasone for 1 hour to induce transcription. Cells then were immediately lysed in a guanidine thiocyanate lysis buffer on the plate. Total cellular RNA was isolated and analyzed by northern blot analysis using digoxygenin-labeled RNA probe complementary to *tk* mRNA.

has been shown for UV photoproducts). We currently are adapting quantitative polymerase chain reaction (QPCR) methods to obtain definitive quantitative data on site-specific damage levels and to directly follow removal of adducts from template DNA in the induced and uninduced state after treatment (or in the absence of treatment) with dexamethasone.

Analysis of the sequence for the LTL region indicates that the *tk* region is highly guanine/cytosine (GC)-rich (65%) containing many tracks of Gs. Other investigators have reported that flanking bases modulate the efficiency of BPDE adduct formation at specific sites and have found higher frequencies of adducts at guanines that occur in tracks of Gs (Lobanenkov et al. 1986). It will be interesting to determine if the inhibition of *tk* transcription is due solely to adducts in the pathway of RNA polymerase on template DNA (elongation), or whether it is influenced by adducts in the promoter region (initiation) required for interaction with the hormone-receptor complex and transcription factors. Initial studies measuring BPDE adducts to DNA in vitro using QPCR indicate higher levels of adducts in the *tk* region compared to those in the LTR, as predicted from sequence analysis (Figure 5). Studies are in progress to measure adduct levels to LTR and *tk* regions of the DNA

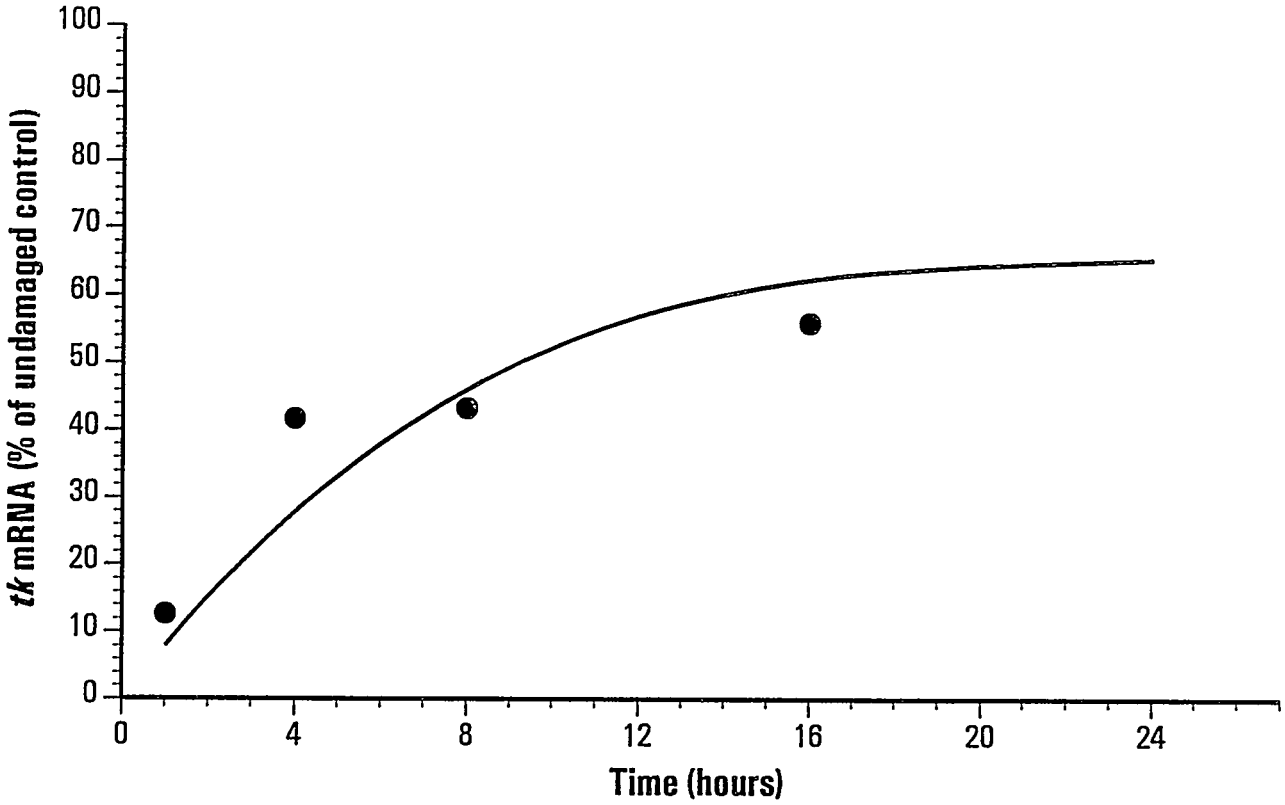

Figure 4. Time Course of Recovery of *tk* mRNA after Treatment with BPDE. Cells were treated in a manner identical to that described in Figure 3 except that a single concentration of BPDE (4.8 μM) was used, and cells were allowed to repair for up to 24 hours in the presence of dexamethasone and 2 mM hydroxyurea (to block replicative synthesis).

in intact cells using QPCR. A similar approach will be used to directly determine rates of repair in these regions. In addition, the technique of ligation-mediated PCR is being adapted to measure adducts at specific sites at the nucleotide level of resolution and the rates of their repair under different transcriptional states (i.e., with and without hormonal induction).

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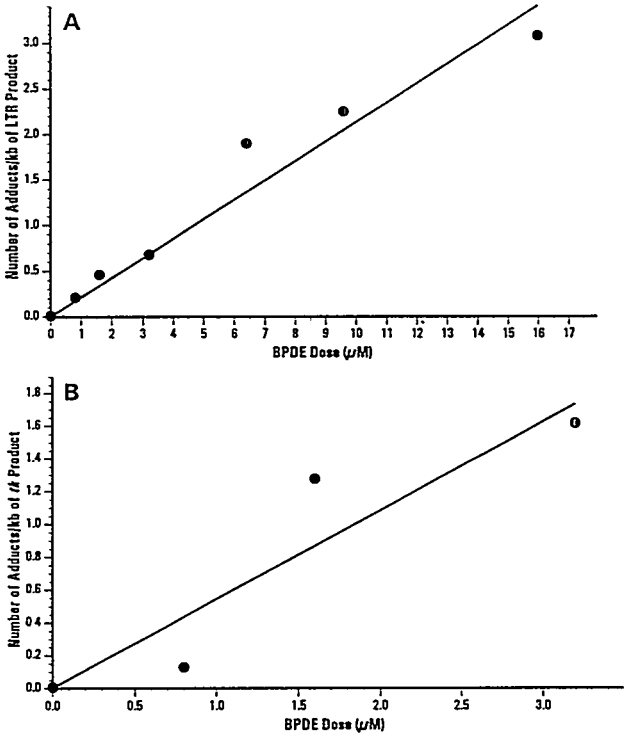

Figure 5. Quantitative Polymerase Chain Reaction (QPCR) Analysis of BPDE Adducts in LTL. Plasmid DNA containing the LTL construct was incubated with various concentrations of (+)-anti-BPDE, extracted with phenol/chloroform to remove reaction degradation products, and precipitated with ethanol. Two nanograms of this template were amplified using primers specific to LTR or the *tk* regions. Unadducted template that amplifies a smaller fragment using the same set of primers was used as an internal control to adjust for efficiency of amplification and gel loading variability. The PCR product was separated on 2% agarose gels and ethidium staining bands were quantified by video densitometry. (A) PCR with primers specific to the LTR region, resulting in a 1.0-kb product. (B) The same template with primers specific for the *tk* gene region, resulting in a 1.4-kb product. (Quantitation of the *tk* product was not normalized to a control fragment.)

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Biochemistry of Free Radical-Induced DNA Damage

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The complementary efforts of characterizing molecular mechanisms underlying free radical-induced DNA damage and developing biochemical methods to examine DNA damage and the rates of repair of such damage in DNA extracted from cells will be important in understanding biological consequences following exposure to energy-related by-products. Differences in levels of DNA damage at critical stages in the cell cycle can result from variations in chromatin structure, in intracellular scavenger and potentiator concentrations, and in rates of repair. Reduced efficiency for repair of damage, whether due to the inability to excise unique types of lesions or due to other factors that modify repair, can result in elevated mutation frequencies which can be correlated with increased tumor incidence. This effort is ultimately aimed at addressing differences in susceptibility of cell populations to energy-related environmental insult, and to uncover the influence of intrinsic or extrinsic factors following such exposure.

Introduction

Mutagenesis, carcinogenesis, and cell death result from a sequence of events initiated by the deposition of ionizing radiation in living cells. DNA damage is a critical step in this process. Despite extensive work focused on developing analytical methods to assess molecular damage to DNA bases, efforts have revealed little information regarding: (1) the spectrum of modifications to DNA occurring in the irradiated cell at nonlethal doses, (2) the potential for transfer of radiation damage along DNA, (3) the spectrum of damage induced as a function of LET, (4) the removal/repair rates of specific DNA lesions, or (5) the efficiency of these lesions at producing the ultimate biological effect. Little information also exists regarding how DNA damage resulting from exposure to ionizing radiation differs from free radical-induced DNA damage caused by oxidative stress, even though both mechanisms produce identical molecular modifications. In this brief review, two areas of work our laboratory has been involved with will be highlighted: our analysis of the molecular mechanisms underlying free radical-induced DNA damage, and our latest efforts to develop biochemical and analytical methods to examine damage in DNA extracted from cells exposed to ionizing radiation. Both areas of our program complement efforts to achieve analysis of relevant types of DNA damage at low exposure doses and to correlate the spectrum of damage observed with biological inactivation. In future studies, we will search for potential differences in the initial production of damage due to intrinsic factors such as chromatin structure, intracellular scavengers (i.e., sulfydryl-containing compounds and polyamines), and intracellular potentiators (i.e., peroxides and trace metals). We also will examine differences in the rates of repair of selected lesions in repair-proficient and repair-deficient cells in an effort to address differences in genetic susceptibility of cell populations to environmental insult.

Highly reactive free radicals are generated by normal metabolic processes in living cells and by exposure to environmental insults such as ionizing radiation. As part of a long-standing effort to understand the mechanistic basis for the biological effects of free radicals on cells, our group continues to be involved in the analysis of free radical-induced DNA damage resulting from exposure to physical (e.g., ionizing radiation and ultrasonic cavitation) and chemical (e.g., hydrogen peroxide) agents. Exposure to ionizing radiation leads to strand breaks (double and single), cross links (DNA-DNA and DNA-protein), and modifications to the deoxyribose and base constituents in DNA and chromatin. We

have intensively studied the product distribution for base damage (Fuciarelli et al. 1989; Fuciarelli et al. 1990), intramolecular cross linking (Fuciarelli et al. 1987; Miaskiewicz et al. in press) and intermolecular cross linking with proteins (Fuciarelli et al. 1987; Weir et al. 1994; Weir-Lipton et al. submitted), and the process underlying electron migration along DNA (see below) in an effort to understand the consequences of exposure, and to establish the mechanistic basis for biological consequences. Although modifications to the purine and pyrimidine constituents in DNA by free radical-generating agents are removed/repaired by specific enzymatic mechanisms, it is possible that the rate of removal/repair of such lesions can vary by orders of magnitude. Understanding which lesions persist in biologically active DNA requires a mechanistic understanding of free radical-induced damage to DNA, as well as development of methods to assay specific types of DNA damage at biologically relevant doses in mammalian cells.

Recent Progress in Understanding the Mechanistic Basis of DNA Damage Underlying Potentially Relevant Biological Endpoints

Free radical-induced damage to DNA following exposure to ionizing radiation is uniquely different in several respects as compared to oxidative DNA damage resulting from normal metabolic processes or from exposure to other physical agents such as ultrasonic cavitation (Fuciarelli et al. 1995) or chemical agents such as hydrogen peroxide (Blakely et al. 1990). The types of free radical species involved are more extensive with ionizing radiation, including both radical ($\cdot OH$, e_{aq}^{-}, $\cdot H$, O_2^{-}) and molecular (H_2O_2, H_2) species. Perhaps the most significant difference is related to the spatial distribution underlying radical formation. Ionizing radiation creates locally multiply damaged sites in DNA, where damage is clustered within a distance of less than 20 base pairs. Other types of oxidative stress (e.g., hydrogen peroxide) result in only singly damaged sites, which are spaced at much greater distances along the DNA (Ward 1991). In cells, singly damaged sites on DNA would be much easier to repair by enzymatic processes than multiply damaged sites such as those generated by exposure to ionizing radiation (and, as we suggested in Fuciarelli et al. 1995, ultrasonic cavitation). Multiply damaged sites in DNA demand a significantly more complex form of enzymatic processing. Damage to both strands of DNA can leave areas of DNA without appropriate template for repair synthesis. DNA repair enzymes may also encounter problems, and stall or fall off the damaged strand, as they attempt to read through extensively damaged areas of the genome.

Damage Characterization: Exposure to Ionizing Radiation Leads to Unique Lesions

Identical DNA base products are formed during normal metabolism (Fuciarelli et al. 1989), and following exposure to ionizing radiation (Fuciarelli et al. 1989, 1990), hydrogen peroxide (Blakely et al. 1990), and ultrasonic cavitation (Fuciarelli et al. 1995). Several different repair enzymes have evolved to eradicate such damage from replicating DNA, thereby preserving genetic integrity. However, DNA damage resulting from exposure to ionizing radiation is also quite unique in two respects. Firstly, classes of DNA damage result from the influence of several different types of radical and molecular products interacting within a very localized area surrounding DNA. Examples of these types of damage include intramolecular cross-linking reactions that lead to 8,5′-cyclodeoxynucleotides (Fuciarelli et al. 1987; Miaskiewicz et al. in press) and intermolecular cross-linking reactions with proteins (Gajewski et al. 1988; Weir et al. 1994; Weir-Lipton et al. submitted) that are characteristically formed following exposure to ionizing radiation and for which repair pathways have not been elucidated. These cross-linking reactions continue to generate interest within our group for three reasons: the mechanism underlying formation of these products is characteristic for exposure to ionizing radiation, the lesions involve structural distortions to the DNA/chromatin complex, and mechanisms for removing/repairing these lesions have not been elucidated.

Secondly, as a result of the high local concentrations of radical and molecular products, DNA

damage resulting from ionizing radiation occurs in multiply damaged areas. This clustering of damage presents repair enzymes with a complex substrate, which is not characteristic of other types of oxidative processes.

In the case of radiation-induced intramolecular cross-linking reactions in DNA, recent molecular modeling efforts (Miaskiewicz et al. in press) complement experimental efforts, thus establishing preference in the stereochemistry leading to formation of isomeric forms of the lesion in DNA. In the case of intermolecular DNA-protein cross-linking reactions, our recent collaborations have led to application of electrospray ionization mass spectrometry to the detection of thymine-tyrosine cross links (Weir et al. 1994; Weir-Lipton et al. submitted). The notable feature of the intra- and intermolecular cross-linked lesions is exemplified in collaborative research efforts by the ability to detect such damage at doses as low as 0.1 Gy in irradiated model systems (Weir-Lipton et al. submitted). Unlike the aforementioned hydroxylated DNA base products, detection of these cross-linked products is not encumbered by relatively high levels of background damage present in DNA extracted from cells with no particular attention to minimizing oxidation.

Our efforts to prevent oxidative damage during DNA isolation from cells is described in this report, and this effort will continue, because post-exposure DNA damage represents a major area of concern regarding analysis of damage in exposed cells. Additionally, exploring new analytical techniques for measuring DNA damage remains a significant avenue of investigation; through collaborative efforts, we are exploring on-line liquid chromatography-electrospray ionization mass spectrometry (Weir-Lipton et al. 1994), and on-line capillary zone electrophoresis- and reversed capillary isotachophoresis-electrospray ionization mass spectrometry (Zhao et al. in press).

Mechanistic Processes: Radiation-Induced Electron Migration Along DNA

Radiation-induced electron migration along DNA is a mechanism by which randomly produced energy deposition events lead to non-random types of damage manifested distal to the sites of the initial energy deposition. Solvated and unsolvated electrons, uniquely generated following exposure to ionizing radiation, can be captured by purine and pyrimidine bases in DNA; these electrons subsequently "tunnel" along DNA in the overlapping pi-electron system created by the stacked bases (reviewed by Fuciarelli et al. 1994b).

Our recent work has led to significant advancements in understanding electron migration in DNA. Radiation-induced electron migration in nucleic acids has been examined using DNA and synthetic oligonucleotides containing 5-bromouracil (5-BrU) (Beach et al. 1994; Fuciarelli et al. 1994a; Fuciarelli et al. 1994b; Fuciarelli et al. submitted[b]). In aqueous solution, interaction of 5-BrU with solvated electrons results in release of bromide ions and formation of highly reactive uracil-5-yl radicals capable of capturing hydrogen atoms from substrates in the irradiated solution. In irradiated solutions of 5-BrU, stoichiometric release of bromide ion and formation of uracil occurs (Fuciarelli et al. submitted[a]); that is, within experimental error all solvated electrons that form during water radiolysis yield bromide ions and uracil. Monitoring either bromide ion release or uracil formation provides an opportunity to study electron migration processes in model nucleic acid systems. Although there is a decrease in reaction rates between electrons and oligonucleotides, as compared to the 5-BrU irradiated as a monomer in solution, 5-BrU is a useful probe of electron interactions in nucleic acids.

Using this approach we have discovered that electron migration along oligonucleotides is significantly influenced by base sequence and strandedness Fuciarelli et al. 1994a). For example, in irradiated single-stranded oligonucleotides the yield of uracil decreased in the following order: $A > T > > C \approx G$. However, in irradiated double-stranded oligonucleotides, the yield of uracil decreased as follows: $G > C \approx T > A$. These differences in the ability of electrons to migrate along DNA were attributed to competing proton transfer reactions occurring within DNA base pairs and between DNA and bulk solvent (Fuciarelli et al. 1994a). Migration along 7 base pairs in oligonucleotides containing guanine bases also was observed for oligonucleotides

irradiated in solution (Fuciarelli et al. 1994a), which compares to average migration distances of 6 to 10 bases for *Escherichia coli* DNA irradiated in solution and 5.5 base pairs for *E. coli* DNA irradiated in cells (Beach et al. 1994). Evidence also suggests that electron migration occurs preferentially in the 5´ to 3´ direction along a double-stranded oligonucleotide containing a region of purine bases adjacent to the 5-BrU moiety (Fuciarelli et al. submitted[b]).

Electron migration is an important process underlying the distribution of radiation damage in DNA, and may be the mechanism causing a nonrandom distribution of DNA damage following energy deposition by stochastic processes. Electron migration at the site of a locally multiply damaged area of DNA can potentiate damage by enhancing the yield of double-strand breaks as a result of migration of radiation damage along one strand of DNA to a position opposite a single-strand break in the complementary strand. Such a mechanism is consistent with the observed enhancement in the radiosensitivity of cells containing 5-BrU-substituted DNA. (For a review see Beach et al. 1994.)

Application of Biochemical and Analytical Techniques for the Assessment of DNA Damage to Cells

To accurately assess initial levels of DNA damage, it is critical to refine techniques to assay free radical-induced DNA damage, with emphasis on developing methods that limit oxidative damage, and to understand repair processing. Moreover, appropriate protocols must be in place to determine the repairability and repair kinetics of specific radiation-induced products from cellular DNA in an effort to investigate the relationship between DNA damage and genetic susceptibility. Evaluation of several different strategies for isolation of DNA from cells exposed to ionizing radiation is occurring now in our laboratory, and development of efficient protocols that will be used to characterize the influence of intrinsic and extrinsic factors influencing repair rates will ultimately result from this important effort.

Reports have indicated that techniques involving phenol extraction potentiate auto-oxidation of nucleic acids (Mouret et al. 1990). Alternative approaches to nucleic acid isolation are therefore necessary to extend assays of cellular DNA damage into the lower dose region. In an effort to achieve more successful protocols, we have compared DNA/chromatin isolation techniques involving phenol/chloroform (Mouret et al. 1990), salt extraction (Gajewski et al. 1990), and precipitation through Qiagen™ columns. Whereas phenol/chloroform extractions generally produced significantly higher background levels of hydroxylated bases, and salt extraction remained a very labor-intensive adventure, isolation of cellular DNA through Qiagen™ columns was rapid (4 hours), consistently quantitative, and potentially the least likely technique for post-exposure hydroxylation of DNA bases. Continued work is necessary, however, to refine this protocol for extraction of cellular DNA for mass spectrometric analysis, with necessary improvements in the sequestration of peroxides potentially liberated during the initial disaggregation of the cells. We also believe that maintenance of a controlled nitrogen atmosphere during subsequent DNA handling will aid in reducing hydroxylated DNA bases.

Future Directions: Combining Mechanistic Knowledge and Technological Developments to Evaluate Individual Susceptibility to Energy-Related Environmental Exposures

Cells deficient in the repair of DNA damage have elevated mutation frequencies; this condition can cause mutated oncogenes or tumor suppressor genes, which potentially leads to increased tumor incidence. The frequency of mutation at any site along DNA is dependent upon damage frequency and the efficiency of repair. Damage frequency is a consequence of (1) physical factors such as energy transfer; (2) chemical factors including intracellular scavengers (e.g., sulfydryl-containing compounds and polyamines) and intracellular potentiators (e.g., peroxides and trace metals); and (3) biological factors such as chromatin structure and repair processing. Our efforts to establish the mechanistic basis for induction of DNA damage and to characterize specific types of

modifications have laid the groundwork for addressing the biological consequences of exposure. In future studies, we will investigate repair rates of selected lesions in repair-proficient and repair-deficient cells in an effort to address differences in genetic susceptibility of cell populations to environmental insult as a result of intrinsic or extrinsic factors. A major first step, which has yet to be categorically achieved and rigorously tested by any group, is measurement of repairability and repair rates of molecular damage in genomic DNA. Reduced efficiency for DNA repair can be the result of 1) unique free radical-induced lesions which challenge the normal complement of repair enzymes; 2) structural characteristics of the lesion(s); 3) spatial distribution of damage (i.e., formation of locally multiply damaged sites); and 4) biochemical conditions which limit efficient repair activity. Ultimately, our goal is to correlate damage production and differences in the rates of repair of selected lesions in cells in an effort to determine whether there are differences in genetic susceptibility to environmental insult.

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Radon Hazards in Homes

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This project identifies and quantifies, in experimental animals, the major biological effects and factors that produce diseases in the respiratory system and other organs in radon-exposed populations in mines and homes. Histopathological analyses have been completed on rats exposed to 40 working-level months (WLM; see footnote d, Table 1) of radon progeny, at 100-working-level (WL; see footnote d, Table 1) concentrations, in combination with uranium ore dust. Changes related to radon-progeny exposure included a 2.4% incidence of primary lung tumors; excluding osteosarcomas, which cannot be unequivocally assigned as primary to the lung, the incidence reduced to 2.0%. Other primary tumors of the respiratory tract were limited to the nose. An updated statistical risk analysis of lung tumors in approximately 2800 exposed rats and 490 controls produced a slightly lower lung-tumor risk coefficient [260 per million rats per WLM (260 per 10^6 WLM)] than reported previously (300 per 10^6 WLM), but still comparable to estimated lifetime risks in underground miners. Because cell proliferation is generally accepted to play a vital role in carcinogenesis, cell proliferation rates in the bronchi-bronchiolar region of Wistar rats were measured following exposure to 174 WLM radon progeny and 0.2-μm-diameter wax carrier aerosols. Preliminary data showed that labeling indices in thoracic airways of exposed rats can be 1½ to 2 times higher than in control rats. The highest ratios occurred in the bronchioles.

Lung cancer incidence and deaths from degenerative lung disease are significant among uranium miners, who often are exposed to high levels of radon, but the cause-effect relationships for these diseases are based on data insufficient to determine risks of public exposures to radon. More recent data on humans suggest that radon also is implicated in other organ diseases, although strong confirmatory data are lacking in animal systems. This project has identified agents or combinations of agents (both chemical and radiological), and their exposure levels, that produced lesions in the respiratory tract and other organs in mine-simulation experiments. The project's current emphasis is on completion of the historical mine-simulation series of experiments, including the low-exposure experiments that also are relevant to radon exposures in homes.

Wistar Rat Exposure Protocols

The 6000 and 7000 Series mine-simulation experiments (Table 1) were designed to develop the relationships between response and exposure to radon progeny, at two rates of exposure, and carnotite uranium ore dust. The radon-progeny exposure rate in the 6000 Series was 1000 working levels (WL) and in the 7000 Series, 100 WL. The 8000 Series mine-simulation experiments (100 WL; Table 2) were designed to extend the exposure-response relationships to cumulative exposure levels comparable to current conditions in uranium mines, and to lifetime environmental exposures. This study continues with a histopathological analysis of the remaining low-exposure animals. The 9000 Series mine-simulation experiments (Table 3) continued the "low-dose" studies at exposure rates comparable to former occupational working levels (10 WL). These experiments help to evaluate the hypothesis that sublinear risk relationships exist at low exposure levels and low exposure rates. In addition, concurrent exposures to varying levels of uranium ore dust also test the hypothesis that irritants (both specific and nonspecific) act synergistically with radiation

Table 1. High Exposure-Response Relationship Study for Radon-Progeny Carcinogenesis in Rats (6000 and 7000 Series Experiments)

Number of Animals[a]			
6000 Series	7000 Series	Exposure Regimen[b,c]	Total Exposure, WLM[d]
64	0	1000 WL[d] radon progeny 15 mg/m^3 uranium ore dust	10,240
56	32	1000 WL radon progeny 15 mg/m^3 uranium ore dust	5120
56	32	1000 WL radon progeny 15 mg/m^3 uranium ore dust	2560
56	32	1000 WL radon progeny 15 mg/m^3 uranium ore dust	1280
88	64	1000 WL radon progeny 15 mg/m^3 uranium ore dust	640
152	128	1000 WL radon progeny 15 mg/m^3 uranium ore dust	320
64	96	Controls	

(a) Number of animals is sufficient to detect the predicted incidence of lung tumors at the 0.05 to 0.1 level of significance, assuming linearity of response between 0 and 9200 WLM and 0.13% spontaneous incidence. Previous exposure at 900 WL for 84 hours/week to 9200 WLM produced an 80% incidence of carcinoma.
(b) Exposure rate, 90 hours/week; planned periodic sacrifice.
(c) Study is repeated at 100-WL (7000 Series experiments) rate (without periodic sacrifice) to augment previous limited exposure-rate data.
(d) Working level (WL) is defined as any combination of the short-lived radon progeny in 1 liter of air that will result in the ultimate emission of 1.3×10^5 MeV of potential alpha energy. Working-level month (WLM) is an exposure equivalent to 170 hours at a 1-WL concentration.

exposures. The exposures of 6000, 7000, and 8000 Series animals are complete. Exposures of 9000 Series animals were suspended with the 80-WLM and 15-mg/m^3 ore-dust exposures to allow analyses of existing data.

Exposures of rats to uranium ore dust alone (10,000 Series experiments; Table 4) are complete. These studies, reported in the *Pacific Northwest Laboratory Annual Report for 1992 to the DOE Office of Energy Research, Part 1*, addressed the potential link of silica exposures to lung cancer. Exposures of rats to radon progeny, uranium ore dust, and cigarette-smoke mixtures are complete. This initiation-promotion-initiation (IPI) study (11,000 Series experiments; Table 5) continues with a histopathological analysis of the remaining IPI animals. Exposures of female rats (12,000 Series experiments; Table 6) are also complete. This study continues with a histopathological comparison to risk data obtained from exposures of male animals using mine-simulation aerosols. Tables 1 through 6 present the actual numbers of animals (including serially sacrificed animals) used at each exposure level. Because the earlier series of experiments were focused more on understanding the influence on risk of radon-progeny unattached fraction and disequilibrium, their protocols are not reported here.

Rat Respiratory Tract Pathology

Histopathology was completed on 8000-Series life-span rats exposed to 40 WLM and 15 mg/m^3 of uranium ore dust. Changes related to radon-progeny exposure included a 2.4% average incidence of primary lung tumors; 1 primary lung tumor was found in control rats (Table 7). Excluding osteosarcomas in the 40-WLM group, which are assumed to be primary to the lung but could have originated elsewhere, the incidence of primary lung tumors reduced to 2.0%. Biologic

Table 2. Low Exposure-Response Relationship Study for Radon Progeny Carcinogenesis in Rats (8000 Series Experiments)

Number of Animals[a]	Exposure Regimen[b]	Total Exposure, WLM[c]
96	100 WL[c] radon progeny, 15 mg/m^3 uranium ore dust	640[d]
396	100 WL radon progeny, 15 mg/m^3 uranium ore dust	320[d]
192	100 WL radon progeny, 15 mg/m^3 uranium ore dust	160
384	100 WL radon progeny, 15 mg/m^3 uranium ore dust	80
480	100 WL radon progeny, 15 mg/m^3 uranium ore dust	40
544	100 WL radon progeny, 15 mg/m^3 uranium ore dust	20
192	Controls	

(a) Number of animals is sufficient to detect lung tumors at the 0.05 to 0.1 level of significance, assuming linearity of response between 0 and 640 WLM and 0.13% spontaneous incidence. Previous exposures indicated a tumor incidence of 16% at 640 WLM.
(b) Exposure rate, 90 hours/week; planned periodic sacrifice.
(c) WL and WLM are defined in footnote d, Table 1.
(d) Repeat exposure is for normalization with Table 1 data.

Table 3. Ultralow Exposure-Rate Study for Radon Progeny Carcinogenesis in Rats (9000 Series Experiments)

Number of Animals[a]	Exposure Regimen[b]	Total Exposure, WLM[c]
64	10 WL[c] radon progeny, 15 mg/m^3 uranium ore dust	320
64	10 WL radon progeny, 3 mg/m^3 uranium ore dust	320
384	10 WL radon progeny, 15 mg/m^3 uranium ore dust	80
384	10 WL radon progeny, 3 mg/m^3 uranium ore dust	80
512	10 WL radon progeny, 15 mg/m^3 uranium ore dust	20
512	10 WL radon progeny, 3 mg/m^3 uranium ore dust	20
192	Controls	

(a) Number of animals is sufficient to detect lung tumors at the 0.05 to 0.1 level of significance, assuming linearity of response between 0 and 640 WLM and 0.13% spontaneous incidence. Previous exposures indicated a tumor incidence of 16% at 640 WLM.
(b) Exposure rate, 90 hours/week; planned periodic sacrifice.
(c) WL and WLM are defined in footnote d, Table 1.

variability in these low-exposure experiments was reflected in the variation ($\pm 100\%$) in primary lung-tumor incidence among the 5 exposure subgroups.

The epidermoid carcinoma in the control rat consisted of numerous large masses of anaplastic squamous epithelium with prominent connective tissue stroma. Although the tumor clearly was potentially fatal, it was not absolutely certain that it originated in the lung; there was, however, no gross description during necropsy that would ascribe its origin to another organ. Other primary respiratory tumors were limited to the nose; 1 exposed rat had an adenocarcinoma and another had a primary malignant lymphoma. No tumors occurred in the larynx or trachea.

Other lesions clearly related to radon progeny and uranium ore-dust exposures included

Table 4. Control Study for Uranium Ore-Dust Carcinogenesis in Rats (10,000 Series Experiments)

Number of Animals	Exposure Regimen[a]
96	15 mg/m^3 uranium ore dust
64	Sham-exposed controls

(a) Exposures, 12 to 18 months at 72 hours/week; planned periodic sacrifice

Table 6. Exposure of Female Rats to Radon Progeny and Uranium Ore Dust (12,000 Series Experiments)

Number of Animals	Exposure Regimen[a]
96	100 WL radon progeny; 640 WLM 5 mg/m^3 uranium ore dust
96	Sham-exposed controls

(a) Exposure rate, 72 hours/week; planned periodic sacrifice.

Table 5. Initiation-Promotion-Initiation (IPI) Protocol for Radon (R), Dust (D), and Cigarette-Smoke (S) Inhalation Exposure of Rats (11,000 Series Experiments)[a]

	Duration of Exposure, weeks					
Group	0	4	8	17	21	25
1	R+D---------->					
2	R+D----->					R+D->
3	R+D---->S---------->					R+D->
4	R+D---------->	S---------->				
5	S---------->			R+D---------->		
6	D---------->	S---------->				

(a) Moderately low concentrations of uranium ore dust accompany radon exposures as the carrier aerosol for radon progeny; sham-exposed control animals (not shown) are included in each exposure group. Ten animals from each exposed or sham-exposed group of 64 rats are killed at 25, 52, and 78 weeks to evaluate developing lesions. Radon-progeny exposures: 100 WL, 320 cumulative WLM; uranium ore-dust concentration: 5 mg/m^3; cigarette-smoke exposures from Kentucky 1R4F cigarettes: 1 hour/day, 5 days/week, for 17 weeks.

accumulations of ore dust in macrophages in the lung and tracheobronchial lymph nodes, and reactive hyperplasia of tracheobronchial lymph nodes. A very low incidence of pulmonary adenomatosis was observed in exposed rats while none was found in the control group; however, this low incidence was within the range found in control groups of previous studies. Also, a low incidence of acute necrotizing pneumonia was present in both control and radon-exposed rats.

The average number of nonrespiratory malignant tumors was higher in exposed rats than in control rats in contrast to 10,000 Series rats exposed to uranium ore dust alone, in which the incidence of nonrespiratory tumors was lower than in control rats. This finding, in part, could be related to the proportionally smaller ratio of control to exposed rats in the 8000 Series 40-WLM rats in contrast to the 10,000 Series uranium ore-dust experiments.

Statistical Risk Modeling

An updated analysis of lung-tumor risks in approximately 2800 exposed and 490 control rats from the 4000 to 9000 Series experiments was initiated in collaboration with Dr. Ethel Gilbert, PNL. The focus of this analysis is to evaluate the modifying factors of exposure rate, age at risk, and time from exposure. A previous analysis (Gilbert 1989) on approximately 750 exposed and 170 control rats included, for the most part, data only from 6000 and 7000 Series rats, and was based primarily on the assumption that all lung tumors were incidental to the death of the animal.

Preliminary data indicate that the overall linear lung-tumor risk coefficient, accounting for tumor fatality, for 20 to over 10,000 WLM

Table 7. Summary of Primary Lung Tumors in Control and Life-Span Rats Exposed to 40 WLM (8000 Series Experiments)

	Exposure Subgroups						Controls
	1	2	3	4	5	Total	
Number of Rats Examined	78	96	95	96	96	461	31
Tumor Type							
Bronchioloalveolar carcinoma	0	0	2	0	1	3	0
Papillary adenocarcinoma	1	0	0	0	0	1	0
Epidermoid carcinoma	0	0	1	0	0	1	1
Adenosquamous carcinoma	0	0	0	1	0	1	0
Undifferentiated carcinoma	0	0	1	0	0	1	0
Bronchioloalveolar adenoma	0	0	0	1	0	1	0
Hemangiopericytoma	1	0	0	0	0	1	0
Osteosarcoma	0	0	0	1	1	2	0
Rats with Lung Tumors, %	2.6	0	4.2	3.1	2.1	2.4	3.2[a]

(a) SPF Wistar rats typically show less than 1% spontaneous incidence of lung tumors.

given at exposure rates ranging from 10 to 1000 WL, is slightly lower (260 per 10^6 WLM) than the previous value (300 per 10^6 WLM); this coefficient is still reasonably comparable to estimated lifetime risks in underground miners (Lubin et al. 1994). A trend was noted for diminishing risks below 320 WLM exposures, but the data were not significant.

Respiratory Tract Cell Proliferation Rates

Studies suggest that cell proliferation is a key parameter in radiation- and chemically induced lung carcinogenesis. Cell proliferation of the bronchi-bronchiolar region of Wistar rats was measured following exposure to radon progeny and a 0.2-μm-diameter wax carrier aerosol. Ninety-one young adult male rats were sham-exposed or exposed whole body to 174 WLM radon progeny at an average 274 WL in 6 consecutive 18-hour exposures. Two control and 5 exposed rats were periodically sacrificed from day 1 to day 84 after start of exposure. To determine cell proliferation rates, rats were implanted subcutaneously with 50 mg bromodeoxyuridine (BrdU), sacrificed after 4 hours, and necropsied for respiratory tract tissues. The tissues were perfused with Carnoy's solution, fixed in alcohol, processed into slides, and stained for BrdU using the Amersham® cell proliferation kit. The percent of labeled nuclei in airway epithelial cells was quantified using light microscopy. Preliminary data show that labeling indices in thoracic airways of exposed rats can be 1½ to 2 times higher than the average in all control rats (Figure 1). Studies to define the kinetics of responses in other damaged regions of the respiratory tract, the regional doses, and the relationships that exist between cell-specific proliferation and cancer induction, are continuing.

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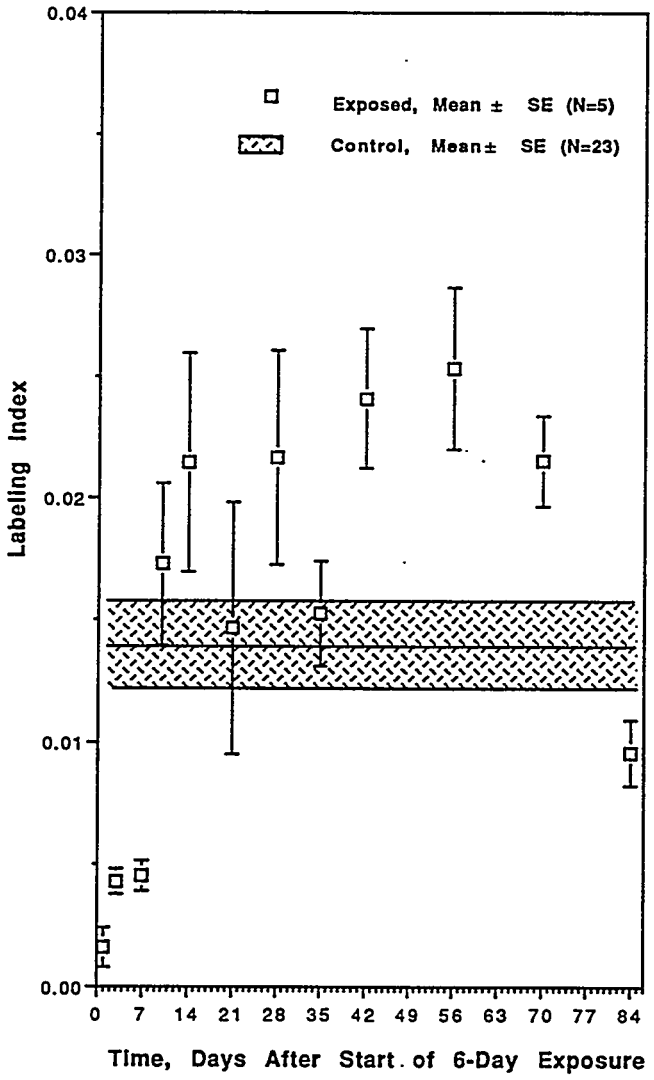

Figure 1. Labeling Index of Rat Bronchial-Bronchiolar Epithelial Cells Following Inhalation Exposure to 174 WLM Radon Progeny and 0.2-μm-Diameter Wax Aerosols

Genetics of Radon-Induced Lung Cancer

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Technical Assistance: R.A. Gies, L.C. Stillwell

Fully assessing the risk of radon-induced lung cancer to the general population will require a complete understanding of the interactions between exposure and genetic factors influencing lung cancer development. Epidemiological investigations indicate a genetic component in human lung cancer unrelated to cigarette smoking. Similarly, the incidence of spontaneous lung cancer varies among genetically distinct inbred mouse strains. Importantly, (1) the risk of radiogenic lung cancer in mice is relative to the strain-specific spontaneous incidence, and (2) the pathogenesis of spontaneous and chemically induced lung tumors in this species is modulated by at least 3 well-defined loci. A pilot study utilizing 2 inbred strains of mice with differing spontaneous rates of lung tumors was begun to test the hypothesis that genetic background modulates the risk of radon-induced lung cancer. If the hypothesis is confirmed, work will be initiated to map the relevant locus (loci). This effort would provide the foundation for cloning the relevant gene(s) for use in defining molecular mechanisms of radon-induced lung carcinogenesis. Also, these genes possibly could be used as probes for polymorphisms that influence risk in human populations. The work also will provide insights for further defining the appropriate epidemiological risk model (i.e., absolute vs. relative) for extrapolating lung cancer risk in radon-exposed miners to the general population.

Background

Genetic background and the environment interact in the pathogenesis of human cancer (Knudson 1991). Familial tendencies and specific disease syndromes that predispose people to cancer have been well-defined (Cox 1994), and direct evidence linking genetic predisposition to cancer induced by radiation in humans is beginning to emerge. Mechanistic studies suggest that the excess skin tumors noted in individuals with xeroderma pigmentosum is a consequence of a cellular defect in the repair of ultraviolet-induced lesions (Cleaver, 1990). It has been noted recently that the excess relative risk of breast cancer in Japanese atomic bomb survivors was higher for early-onset disease than that for cancer diagnosed in older women (Tokunaga et al. 1994), suggesting interactions between radiation exposure and well-established genetic predisposition to pre-menopausal breast cancer.

Although little is known regarding genetics of radiation- or radon-induced lung cancer, there is epidemiological evidence indicating genetic predisposition to spontaneous disease as well as polymorphisms affecting sensitivity to smoking-induced cancer. Studies have shown that lung cancer risk in particular (Samet et al. 1986; Ooi et al. 1986) or overall risk of cancer in general (Lynch et al. 1986) not related to cigarette smoking is increased in relatives of lung cancer patients. Essentially, all small-cell lung cancer and ~50% of non-small-cell lung tumors exhibit deletions in chromosome regions 3p(14-23); cytogenetic abnormalities in this region have been shown to co-segregate with renal carcinoma. The region also is believed to contain a fragile site (reviewed in Minna 1990). These

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observations suggest a tumor suppressor gene reminiscent of retinoblastoma.

Molecular studies have shown a germ-line polymorphism in the *p53* gene associated with increased lung cancer risk (Kawajiri et al. 1993). The observation is controversial because others have not found a relationship between *p53* polymorphisms and lung cancer in other populations (Weston et al. 1992). A variety of polymorphisms in enzymes affecting carcinogen metabolism have been identified; these polymorphisms modulate susceptibility to smoking-induced lung cancer (reviewed in Idle 1991).

Inbred mice provide a powerful tool for characterizing the effects of genetic background on the pathogenesis of lung cancer. The spontaneous incidence of lung tumors in strains varies widely; C57BL/6J (B6) mice infrequently develop lung tumors (~7%) whereas essentially all A/J (A) mice have multiple lung tumors by 6 months (Hahn 1993). Moreover, exposure to a variety of inhaled or injected chemical carcinogens results in a proportionately greater number of tumors in A mice than in B6 mice (Hahn 1993). These observations suggest that risk of lung neoplasia in this species is relative to the genetically defined spontaneous rate as opposed to a simple increase in the the absolute number of tumors as a function of carcinogen dose.

The risk of spontaneous and chemically induced lung tumors in this species is controlled by 3 loci designated *Pas* (pulmonary adenoma sensitivity) genes. Through molecular analysis, 1 of these genes has been identified as a polymorphism in the K-*ras2* proto-oncogene (Ryan et al. 1987). Another *Pas* gene has been found to be closely linked to the mouse H-2 complex (reviewed in Demant et al. 1989). The correlation between increased proliferation indices of lung epithelial cells and cancer susceptibility in inbred mouse strains has led to speculation that the third *Pas* gene is involved in regulating the growth or differentiation of lung-tumor precursor cells (Malkinson 1991). In addition to the *Pas* genes, genetic studies indicate the presence of a mouse gene mapping to chromosome 4 that acts as a lung cancer suppressor gene in this species (Herzog et al. 1994).

Mice have been shown to be susceptible to radiogenic lung cancer. Exposure to external X-rays and neutrons induces lung tumors in inbred BALB/c mice (Ullrich 1983, 1984) and outbred SAS/4 mice (Coggle 1988). More relevant to radon exposure is the observation that alpha particles from inhaled $^{239}PuO_2$ induce lung tumors in B6 mice, with protraction of the alpha-particle dose increasing the carcinogenic risk (Lundgren et al. 1987). A large-scale analysis of cancer-incidence data obtained using C3hf/Bd, C57BL/6Bd, BALB/c, and RFM mice exposed to external X-rays and ^{137}Cs gamma rays indicates that (1) the general susceptibility to radiogenic cancers of the lung is correlated with species-specific spontaneous incidences, and (2) the relative risk estimate in mice for radiogenic cancer of the lung and breast as well as for leukemia was not significantly different from that in humans (Storer et al. 1988).

Historically, mice have not been employed extensively in quantifying radon risk. Studies at the University of Rochester in the late 1960s suggested that mice are refractory to radon-induced lung cancer (Morken 1973). However, the animals were exposed to what would now be considered very high cumulative exposures (the lowest estimated to be 18,000 WLM) at very high exposure rates. At roughly the same time, similar results were obtained with B6 mice exposed to very high radon levels at Pacific Northwest Laboratory (Palmer et al. 1973).

The relevance of these studies is questionable for several reasons. Subsequent studies with rats, which are susceptible to radon-induced lung cancer, have revealed an inverse exposure-rate effect in which carcinogenicity increases with decreasing exposure rate (Cross 1991). Given the very high exposure rates, the significant life-span shortening, and the amount of radiation-induced cell death of airway epithelium noted in both sets of early studies with mice, it is not surprising that lung tumors were not noted. The current studies were designed to assess tumor incidences at lower cumulative exposures levels comparable to those which have been shown to induce lung cancer in rats.

Experimental Design and Preliminary Results

The specific aim of this pilot study is to determine if the mouse genes that modulate the patho-

genesis of spontaneous and chemically induced lung neoplasms also modulate the neoplastic response to inhaled radon and progeny. The A and B6 strains were chosen for the reasons described in the previous section. If genetic background is found to affect radon-induced lung-tumor development, the long-term goal of the project will be to locate and clone the relevant gene(s) using either recombinant inbred strains obtained from A and B6 parent lines or simple sequence length polymorphisms associated with lung neoplasms in backcross progeny of A x B6 F1 animals bred with B6 animals.

For the current study, subgroups from each strain were exposed to 1000 and 2500 WLM (for a definition of WLM, please see the report by Cross, this volume) radon progeny (nominal) using a uranium ore-dust carrier (carnotite, ~5 mg/m^3) at an exposure rate of ~110 WLM/day (Table 1). The exposures were interrupted daily for routine animal care; they were performed on consecutive days without weekend breaks. Control animals were exposed to ore dust only. As positive controls, additional animals from each strain were injected intraperitoneally with ethyl carbamate (1 mg/g body weight) using physiological saline as a vehicle.

The primary data to be collected from all groups are lung-tumor incidence and multiplicity as a function of time. Ancillary data will include a complete necropsy for extrapulmonary lesions; histological evaluation of random lung tissue; lung lavage for total cells, differential counts, and total protein; and lung epithelial cell cytodynamics as measured by indices of cell birth and death. The latter will include quantifying in situ the fraction of proliferating deep-lung epithelial cells using Proliferating Cell Nuclear

Table 1. Experimental Design for the Pilot Study to Test the Hypothesis that Genetic Background Modulates the Risk of Radon-Induced Lung Cancer

			Sacrifice Schedule (animals/group)		
Strain	Agent	Exposure	30 weeks	60 weeks	90 weeks
A/J	none	-	-	-	15
	ethyl carbamate	1 mg/g body wt	10	10	15
	radon progeny[a]	1000 WLM[b]	10	10	15
	ore dust only[a,c]	-	10	10	15
	radon progeny	2500 WLM[b]	-	-	15
	ore dust only[d]	-	-	-	15
C57BL/6J	none	-	-	-	15
	ethyl carbamate	1 mg/g body wt	10	10	15
	radon progeny[a]	1000 WLM[b]	10	10	15
	ore dust only[a,c]	-	10	10	15
	radon progeny	2500 WLM[b]	-	-	15
	ore dust only[d]	-	-	-	15

(a) Divided between 2 separate exposures.
(b) Nominal cumulative exposure.
(c) Matched for the nominal 1000-WLM cumulative exposures.
(d) Matched for the nominal 2500-WLM cumulative exposure.

Antigen (PCNA, clone PC10) staining, and quantifying the fraction of cells undergoing apoptosis based on the section of fragmented nuclear DNA using a commercially available apoptosis detection kit (ApopTag™, Oncor). A collaboration has been established with Dr. Roger Thrall of the University of Connecticut Health Center to evaluate any non-neoplastic changes in the lung epithelium.

To ensure that no subtle anatomical differences between strains affected the dose to the lung epithelium despite equivalent radon and progeny exposure levels, additional animals from each strain were exposed to ~300 WLM radon and progeny; micronuclei frequency in deep-lung fibroblasts were quantified using the method of Khan et al. (1994). Similar data were obtained for control, ore dust-only, and ethyl carbamate-injected animals. There were no observed differences between strains in the amount of scored, cytogenetic damage in deep-lung fibroblasts obtained from animals from all 4 treatment groups (Figure 1). Interestingly, micronuclei frequency in the cells obtained from animals exposed to ~300 WLM radon was approximately 3 times that noted for cells obtained from ethyl carbamate-treated animals.

The direction of future work is dependent upon the outcome of the pilot study. As mentioned, if the incidence of radon-induced lung neoplasms is found to be strain-dependent, future work will be aimed at mapping the relevent locus (loci). If the mouse genes influencing the pathogenesis of spontaneous and chemically induced lung tumors are found not to modify the tumorigenic effect of radon, cross-species comparisons between mice and sensitive species as well as in vitro work to identify potential differences will be initiated.

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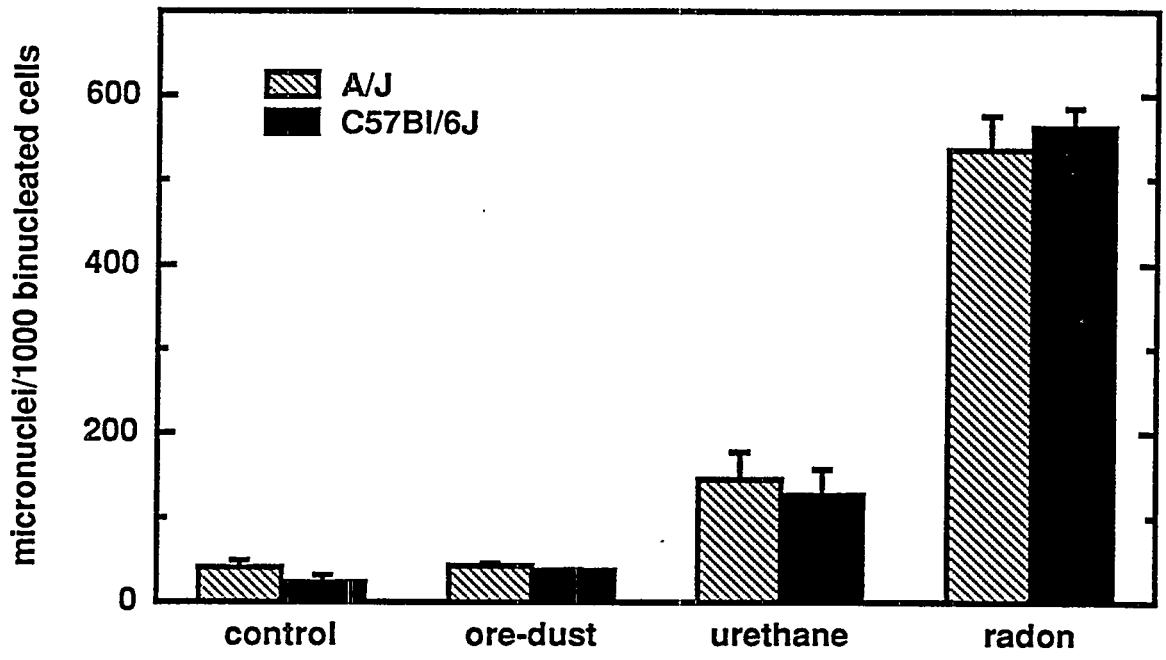

Figure 1. Micronuclei Frequency (±SD) in Deep-Lung Fibroblasts Obtained from Mice 24 Hours after Ethyl Carbamate Injection (urethane, 1 mg/g body weight) or Exposure to 300 WLM (nominal) Radon Progeny

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Mechanisms of Radon-Induced DNA Damage

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Other Investigators: A.L. Brooks, F.T. Cross, T.E. Hui, L.H. Lutze,[a] R.C Miller,[b] J.M. Nelson, and J.P. O'Neill[c]

Technical Assistance: R.A. Gies, L.C. Stillwell

In this project, we conduct dosimetric, molecular, cellular, and whole-animal research relevant to understanding the mechanisms of radon and radon-progeny injury to DNA. The work specifically addressed the nature of radon-induced mutations in (1) the *LacI* gene obtained from the lung tissue of Big Blue™ transgenic mice exposed in vivo; (2) human lymphocytes exposed in the Pacific Northwest Laboratory (PNL) in vitro exposure system for molecular analysis of mutation; and (3) C3H 10T½ cells exposed in the PNL in vitro exposure system for transformation analysis. Southern blot and polymerase chain reaction (PCR) exon analysis of radon-induced Chinese hamster ovary (CHO)-HPRT mutations were completed and the study published in *Radiation Research* (Jostes et al. 1994). The gross molecular spectrum of radon-induced mutations showed a marked increase in the frequency of deletions relative to that observed in the spontaneous spectrum, but no difference was noted between the molecular spectra of mutations induced by radon and those induced by 300-cGy x rays. We are currently investigating the fine structure of radon-induced mutations in the *LacI* gene isolated from lung tissue of a Big Blue™ transgenic mice.

In Vivo Radon Studies

We have exposed Stratagene Big Blue™ transgenic mice by inhalation to 320, 640, and 960 working-level months (WLM) of radon progeny. Mice were sacrificed after 3, 6, and 9 days, the time periods required to obtain the exposures. Control mice also were sacrificed at the same time intervals. Mutations were scored only if they occurred *unambiguously* in both strands of the mutant gene; the gene then was evaluated in its entirety. Induced mutation frequencies increased with exposure level and were 3 to 4 times spontaneous frequencies. Twelve *LacI* mutations were isolated from the lung tissue of a mouse from the 960-WLM group, and the *LacI* gene has been sequenced. In addition, 16 *LacI* mutations were isolated from the lung tissue of a mouse from the 640-WLM group; 7 have been completely sequenced. Nine *LacI* mutations from the lung tissue of unexposed control mice also were sequenced.

Sequence data from the unexposed mice were similar to that found in lung tissue at Stratagene; predominantly G:C to A:T transitions in the protein-associated region (Table 1). The mutation spectrum from radon-exposed mice was markedly different from that obtained from the control mice. Small deletions and insertions constituted 53% of the mutations in the radon-exposed mice; only 1 was in the protein-associated region of the gene. Six of the mutations (26%) obtained from exposed mice exhibited multiple events within the gene. In some, deletions, insertions, and base changes occurred together in the same gene. This observation is consistent with the concept of locally multiply damaged sites after alpha irradiation. No multiple events were noted in the

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(b) Columbia University
(c) University of Vermont

Table 1. Types of Mutagenic Events in Spontaneous and Radon-Induced *LacI* Mutants

	Spontaneous Mutants, %		Radon-Induced Mutants, %
	Stratagene[a]	PNL	PNL
Transitions			
A:T to G:C	0% (0)[b]		3% (1)
G:C to A:T	56% (5)	67% (6)	23% (7)
Transversions			
G:C to T:A	22% (2)	11% (1)	10% (3)
G:C to C:G	11% (1)	11% (1)	0% (0)
A:T to C:G	0% (0)		10% (3)
A:T to T:A	0% (0)		0% (0)
Other (insertions & deletions)	11% (1)	11% (1)	54% (16)
Total	100% (9)	100% (9)	100% (30)

(a) Stratagene 1994.
(b) Numbers in parentheses indicate actual number of mutagenic events in sequenced mutations.

spontaneous mutations evaluated to date. Figure 1 illustrates the shift from predominantly base-mispairing events in the spontaneous mutants to deletions and insertions in the radon-exposed mice.

Mutational events in radon-exposed mice are approximately equally distributed throughout the gene, in contrast to the spontaneous mutations, in which half of the mutations are clustered in the protein-associated region of the gene (Figure 2). The break-point rejoining regions of large deletions obtained from radon-exposed mice are being investigated collaboratively with Dr. Louise Lutze at the University of California, San Francisco. These studies will help define the molecular fine structure of mutations induced by high-LET radiation exposure.

In Vitro Radon Studies

Pacific Northwest Laboratory (PNL)

Three studies are in progress at PNL:

In vitro cell exposure system. The PNL in vitro radon cell-exposure system was employed in PNL experiments as well as in several collaborative experiments with other laboratories. The collaboration with Dr. E.W. Fleck, Whitman College (Walla Walla, Washington), on molecular analysis of radon-induced CHO-HPRT mutations using Southern blot and polymerase chain reaction (PCR) exon analysis was completed and published in *Radiation Research* (Jostes et al. 1994). The gross molecular spectrum of radon-induced mutations showed a marked increase in the frequency of deletions relative to that observed in the spontaneous spectrum, but no difference was noted between the molecular spectra of mutations induced by radon and those induced by 300-cGy x rays. A clustering of deletion breakpoints was noted in the 3′ half of the *hprt* gene (spontaneous and irradiated).

Fluorescent in situ hybridization. A collaboration with Dr. Brooks at PNL investigating the occurrence of color junctions in chromosomes prepared using fluorescent in situ hybridization after radon and x-ray exposures has been completed, and a manuscript has been submitted to *Environmental and Molecular Mutagenesis*. This work is described in this Annual Report under "Genotoxicity of Inhaled Energy Effluents."

Microbeam. A project investigating chromosome aberration induction using the PNL microbeam has been initiated. A limited number of mitotics from asynchronous CHO cells given

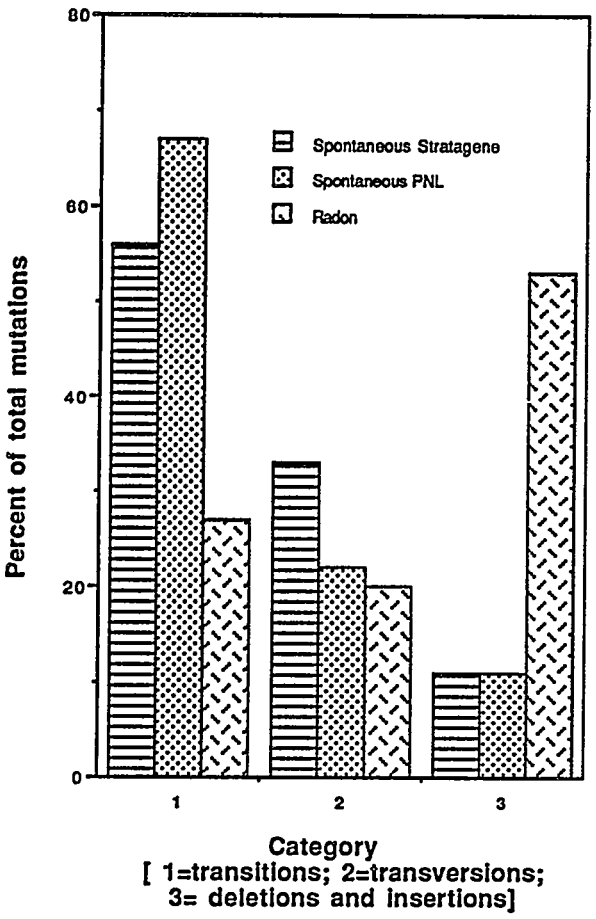

Figure 1. Histogram Illustrating the Relative Frequency of Transitions, Transversions, and Deletions/ Insertions in Spontaneous and Radon-Induced *Lacl* Mutations

1 alpha particle per nucleus have been scored. These preliminary data indicate that 37% of the metaphases from the irradiated cells contained aberrations.

PNL/University of Vermont

In a collaborative study with Drs. J.P. O'Neill and R.J. Albertini at the University of Vermont, we are investigating cell survival and mutation induction in human peripheral blood lympho-cytes after in vitro radon exposure. Dose-response data was evaluated; mutants will be isolated from lymphocytes exposed to a dose between 30 and 40 cGy, a range that yields a single alpha traversal probability of 32 to 36%. Two additional exposures were completed in FY 1994, and in the future molecular analysis of isolated mutants will be performed.

PNL/Columbia University

The collaboration with Drs. Richard Miller and Eric Hall (Columbia University), in which C3H 10T½ cells were exposed in PNL's in vitro radon exposure system, were completed. Cell survival and transformation were evaluated at 3 dose levels in 2 experiments. Technical help for transformation experiments at PNL was provided by Columbia University. The D_0 was established at approximately 50 cGy (representing 3 alpha traversals per cell nucleus; 2.1 x 10^{-3} keV total energy deposited in the nucleus). Transformation frequencies obtained from PNL's in vitro radon exposure system were similar to those obtained from 90- and 120-keV/mM alpha particles generated in the Columbia University Radiological Research Accelerator Facility (RARAF). A manuscript describing this work has been prepared for submission to *Radiation Research.*

A comparison of suspension-exposure systems versus planar-exposure systems with regard to dose (cell nucleus) was accomplished by calculating the average numbers of alpha traversals at the D_0. We determined that the average traversal number required to achieve D_0 in C3H 10T½ cells increased as the average traversal distance decreased (such as from flattened nuclei in the planar irradiation geometry) . As expected, the average total energy deposited in the cell nucleus at D_0 was approximately the same in planar and spherical geometries (1.6 - 2.1 x 10^{-3} keV). Table 2 presents selected data illustrating this relationship. These findings were presented at the 1994 Annual Meeting of the Health Physics Society.

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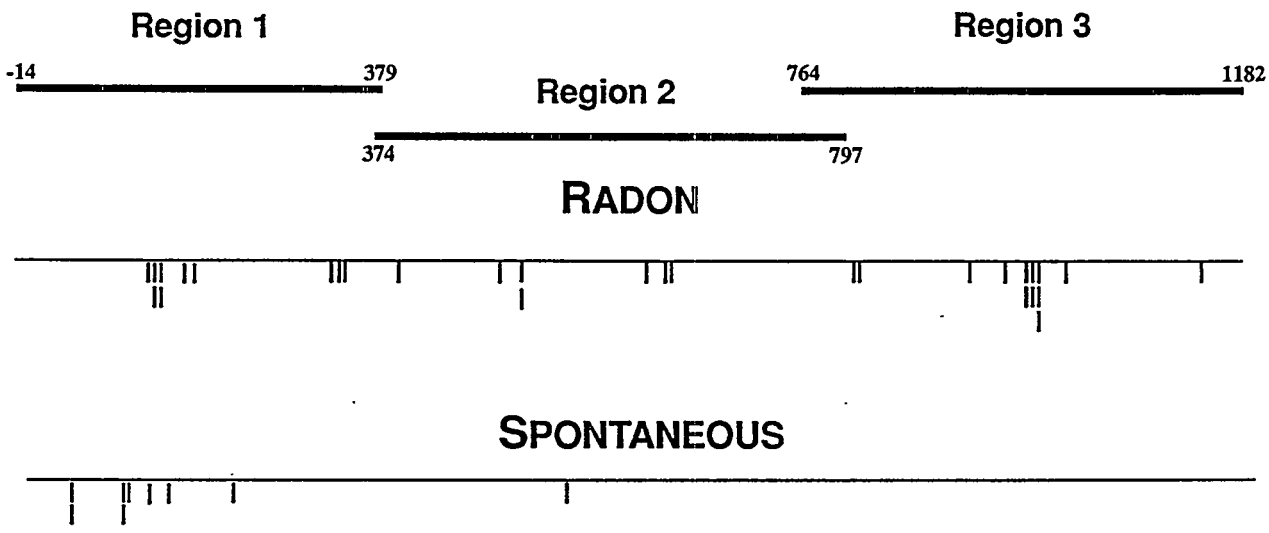

Figure 2. Distribution of Mutations in the *LacI* Gene Obtained from Radon-Irradiated and Spontaneous Lung Tissue. Tick marks indicate the location of mutations; stacked marks indicate the same mutation in the same base pairs.

Table 2. Comparison of Alpha Traversals and Energy Deposited per Nucleus at the D_o

Study	Cell	Exposure System	D_o (cGy)	Track Length[a]	LET (keV/μM)	Alpha Traversals at the D_o	Energy Deposited[b] (keV)
Jostes et al. (unpublished data)	C3H 10T½	Suspension	50	6.6	103	3.1	2.1×10^3
Raju et al. 1991	C3H 10T½	Collimated planar	60	2.1	121	6.4	1.6×10^3
Lloyd et al. 1977	C3H 10T½	Collimated planar	60	~1.7 (estimated)	~85 (estimated)	13.8	2.0×10^3

(a) Average track length (in mm) of each alpha traversal of the nucleus.
(b) Average total energy deposited in the cell nucleus at the D_o.

Raju, M.R., Y. Eisen, S. Carpenter, and W.C. Inkret. 1991. Radiobiology of alpha particles. III. Cell inactivation by alpha-particle traversals of the cell nucleus. *Radiat. Res.* 128(2):204-209.

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In Vivo/In Vitro Radon-Induced Cellular Damage

Principal Investigator: A.L. Brooks

Other Investigators: M.A. Khan, F.T. Cross, K.M. Groch, and R.F. Jostes

To understand the health effects of inhaled radon and its progeny, this project provides important links relating the data from mechanistic model studies to those derived from animal studies, and renders both types of data more useful in predicting health hazards from radon-progeny exposure in homes. In this project, we compare cytogenetic damage induced by high-LET alpha particles and low-LET gamma rays given both in vivo and in vitro to define the relationships between working-level months (WLM) from radon-progeny inhalation exposure and radiation dose to cells of the respiratory tract. During FY 1994, we investigated the influence of biological and physical variables on this exposure-to-dose relationship, and determined the radon relative biological effectiveness (RBE) both in vivo and in vitro. Experiments were directed toward (1) understanding the influence of species/strain on the induction of micronuclei by radon-inhalation exposure; (2) evaluating the role of dose rate on chromosome damage from both high- and low-LET radiation exposure; and (3) investigating the influence of radon-progeny aerosol characteristics on respiratory tract deposition, dose, and induced damage.

Animals and cultured cells were exposed to radon and its progeny; cellular damage was determined in deep-lung fibroblasts using the micronucleus assay. The sensitivity of different species or strains to the induction of cancer was not reflected in an increase in the sensitivity to micronuclei induction in primary lung fibroblast cultures. However, marked species differences were observed in the frequency of micronuclei induced per unit of exposure. The frequency of induced micronuclei decreased as a function of dose rate for low-LET radiation exposure but showed an increase with decreasing dose rate for radon exposure. The dose-rate effectiveness factor was 6.0 when comparing the frequency of micronuclei induced following an acute radon exposure with that from a 67-hour protracted exposure. By comparing micronuclei induction at equal doses of ^{60}Co and radon delivered in an equal time period, we obtained a high RBE of about 65. The damage per WLM exposure from radon associated with wax and uranium ore-dust aerosols was higher than that associated with cigarette smoke aerosols.

This research addresses basic radiobiologic questions associated with radon inhalation (herein taken to mean "radon and its progeny") and helps provide a mechanistic basis for understanding the action of radon on respiratory-tract cells. This information provides the basis for better estimates of risk from indoor radon exposure. Key questions we're addressing are:

1. Is there a relationship between the genetic susceptibility of animals for the induction of lung cancer and the sensitivity of the lung cells to the induction of micronuclei?
2. What is the dose-rate effectiveness factor for protracted low-LET irradiation exposure, and, at low dose-rate exposures, what is the relative biological effectiveness (RBE) for radon-induced damage with respect to protracted low-LET radiation?
3. How does the radon-progeny carrier aerosol influence the distribution of dose and damage in deep lung fibroblasts?

Experimental Design and Methods

To evaluate the relationship between the sensitivities of animals to cancer and micronuclei induction, studies were conducted with different rodent species and strains: Wistar rats, Syrian Golden hamsters, Chinese hamsters, C57BL/6J mice, and A/J mice. The rat is very sensitive to radiation-induced lung cancer while the Syrian Golden and Chinese hamsters are not as sensitive. The C57BL/6J mouse is resistant to

the induction of lung cancer by urethane, whereas the A/J mouse is sensitive to lung cancer induction by this chemical. Our hypothesis is that sensitivity to lung cancer induction by either radiation or chemicals would be reflected by an increase in the frequency of radon-induced micronuclei or by a decrease in radon-induced repair of cytogenetic damage. To evaluate cytogenetic damage and repair, the frequency of induced micronuclei in lung fibroblasts was measured using the methods of Khan and Heddle (1992). Animals were exposed to radon using the in vivo system developed by Cross et al. (1984). This study addressed 2 questions: (1) Is initial damage between species the same? (2) Is the damage handled biologically the same in each species over time? All exposures were conducted with the same carrier aerosol (uranium ore dust) and the dose and fractionation time (3 to 5 days) was similar for each species. For the between-species and strain comparisons, animals were sacrificed 4 hours after the end of the radon exposure. Thereafter, at 4 hours and at 15 and 30 days after radon inhalation, additional rats, Syrian Golden hamsters, and Chinese hamsters were sacrificed to evaluate the loss of micronuclei from the cell population. The cells were harvested as described by Khan et al. (1994) and placed in culture for 72 hours. The cultures were treated with cytochalasin B to block cytokinesis, then micronuclei frequency was determined.

In another study, the limiting dose-rate effect for low-LET radiation and the RBE for radon relative to low-LET radiation were evaluated in Wistar rats. Rats were exposed to 0, 1, 2, and 4 Gy of ^{60}Co gamma rays delivered over 20 minutes. Two additional groups of animals were exposed to 0, 4, 8, and 12 Gy delivered over either 4 or 67 hours. The 2 protracted exposures matched the times required for the radon exposures above. Animals were sacrificed 4 hours after the end of each exposure and the irradiated fibroblast cells evaluated for the induction of micronuclei as described above.

The influence of different carrier aerosols was determined by evaluation of induced micronuclei in rat lung fibroblasts following inhalation of radon progeny attached to wax, uranium-ore dust, or cigarette smoke. In all studies, the cells were scored for micronuclei induction on coded slides.

Results and Discussion

Species/Strain Study

The exposure-response relationships for in vivo induction of micronuclei by radon inhalation in Wistar rats and Syrian Golden hamsters have been reported (Khan et al. in press). The comparison between exposure-response relationships for Wistar rats and Syrian Golden hamsters is shown in Figure 1. These relationships were fit by the least-squares method to linear equations (micronuclei/1000 binucleated cells = 15.5 ± 14.4 + 0.53 ± 0.06 WLM for the rat; micronuclei/1000 binucleated cells = 38.1 ± 15.1 + 0.80 ± 0.08 WLM for the Syrian Golden hamster). There was a single data point at 500 WLM for the Chinese hamster, which resulted in 1.83 ± 0.02 micronuclei/1000 binucleated cells/WLM. These observations are particularly interesting, because the Wistar rat is much more sensitive to radon-induced lung tumors than the Syrian Golden hamster (Cross

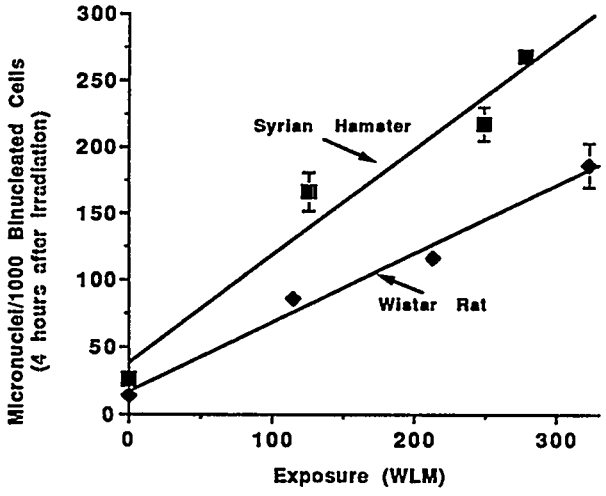

Figure 1. Comparison of Exposure-Response Relationships for Radon-Induced Micronuclei Between Wistar Rats and Syrian Golden Hamsters. Slope estimate for the Syrian Golden hamster was significantly higher ($P < 0.05$) than that estimated for the Wistar rat.

et al. 1981). The current data show that, per WLM, more damage is present in the hamster lung than in the rat, and suggest that differences in sensitivity to the induction of lung cancer may not be related to the amount of initial chromosome damage induced, because this type of damage is usually lethal. The results of the mouse study are plotted in Figure 2. There were no significant differences observed in the frequency of micronuclei induced by radon in the 2 different strains of mice, even though A/J mice are much more sensitive to chemically induced lung cancer. The frequency of induced micronuclei per unit exposure was 1.11 ± 0.05 and 1.12 ± 0.03 micronuclei/1000 binucleated cells/WLM for the A/J and C57BL/6J mice, respectively. This induced frequency is similar to that observed in the Chinese hamster and illustrates that the mouse is also less sensitive to the induction of initial genetic damage than the rat. It may be necessary to grow epithelial cells from the different species to resolve these differences.

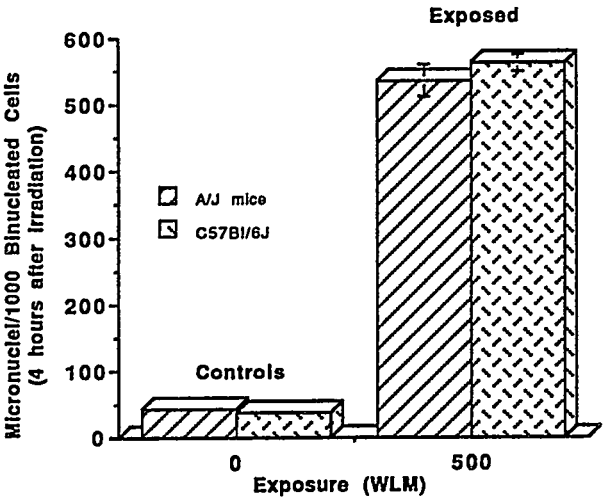

Figure 2. The Induction of Micronuclei by Radon Inhalation in the Deep-Lung Fibroblasts of 2 Different Strains of Mice (A/J and C57BL/6J). There were no significant differences observed between the two strains.

To evaluate the persistence of cytogenetic damage in the cell population, a comparison of the relative sensitivity per WLM of Wistar rats, Syrian Golden hamsters, and Chinese hamsters at all 3 sacrifice times was reported (Khan et al. in press); this comparison is shown in Figure 3. This figure suggests that the rat is the least sensitive of the 3 rodents, based on initial and sustained cytogenetic damage, and illustrates that the rate of loss of damage from the cell population is very similar for the rat, and Chinese and Syrian Golden hamster. These data further suggest that the between-species differences in sensitivity to cancer induction is not related to the rate of loss of the cells that contain cytogenetic damage from the cell population. This loss is probably related to cell death and cell proliferation, and seems not to be related to cancer induction sensitivity.

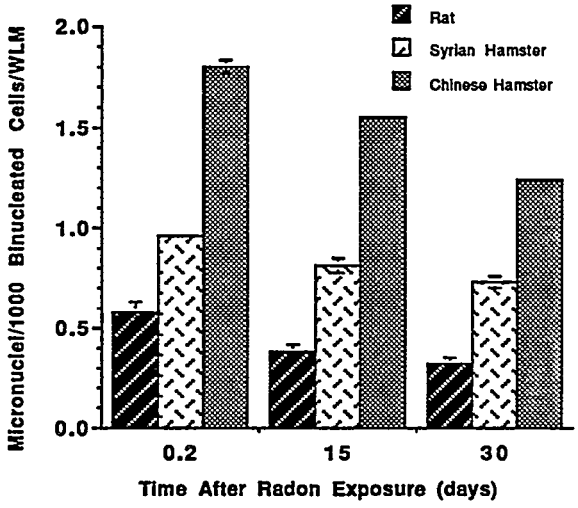

Figure 3. Comparison of Radon-Induced Micronuclei Frequency per WLM in Wistar Rat, Syrian Golden Hamster, and Chinese Hamster Lung Fibroblasts as a Function of Time (4 hours, and 15 and 30 days) Between the End of Radon Exposure and Sacrifice

Relative Biological Effectiveness Study

To determine an RBE that is relevant to environmental radon exposures, it is necessary to develop low- and high-LET dose-response relationships at low dose and dose rates. We previously reported on the comparison of micronuclei induction for high dose-rate ^{60}Co gamma rays and radon, both in vivo and in vitro (Brooks et al. in press). The reported in vitro values reported indicate that radon is 10.9 ± 2.6 and 12.5 ± 2.4 times as effective as ^{60}Co in inducing micronuclei in primary rat lung fibroblasts and CHO-K1 cells, respectively. For lung fibroblasts exposed by radon inhalation or by ^{60}Co gamma rays in vivo, radon was 10.6 ± 1.0 times as effective as acute whole-body ^{60}Co exposure. To make RBE comparisons that are more valid at environmental levels, where the dose rates are very low, it is necessary to establish the limiting dose rate for the exposure systems, especially for the low-LET radiation source. Figure 4 shows the dose-response relationship for the induction of micronuclei in vivo by ^{60}Co delivered over 3 different times. The dose-response relationships were fit with a linear least-squares analysis; they decreased as a function of increased exposure time. The equations that described the dose-response relationships (where D = dose in Gy) were micronuclei/binucleated cell = 0.01 + 0.060D for acute exposure; micronuclei/binucleated cell = 0.02 + 0.024D for exposures protracted 4 hours; and micronuclei/binucleated cell = 0.007 + 0.010D for exposures protracted 67 hours. If the damage produced by ^{60}Co and radon delivered over the same time are compared, the RBE value is estimated to be 65. The dose-rate effectiveness factor for the induction of micronuclei was 6.0 when the highest dose rate was compared to the lowest dose rate.

Carrier-Aerosol Study

Studies have been conducted to determine if changing the radon-progeny carrier aerosol will change the dose and damage in the deep lung per WLM of exposure. Rats were exposed to similar total WLM exposure in association with a cigarette-smoke aerosol, a 0.2-mm-diameter wax aerosol, and a uranium ore-dust aerosol.

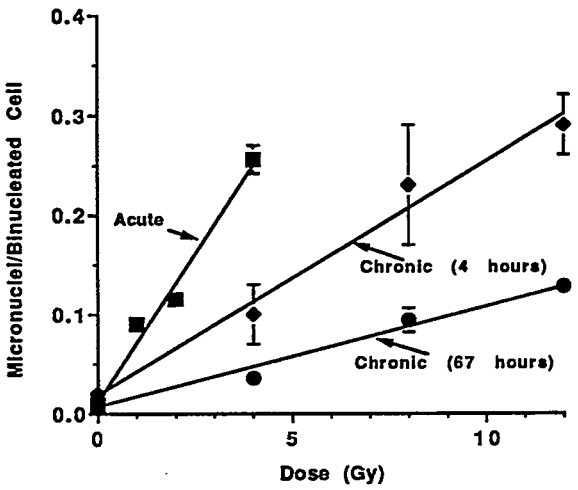

Figure 4. The Influence of Dose Rate on ^{60}Co-Inducted Micronuclei in Deep-Lung Fibroblasts Exposed In Vivo

Figure 5 illustrates that the wax aerosol was the most effective (1.5 micronuclei/1000 binucleated cells/WLM) in inducing micronuclei in the deep lung, followed by the ore dust (0.47 micronuclei/1000 binucleated cells/WLM) and the cigarette-smoke aerosol (0.30 micronuclei/1000 binucleated cells/WLM). It should be noted that the dose rate of the animals exposed to the smoke aerosol was higher than that of the other 2 aerosols. The uniform size of the wax aerosol (0.2 μm) seems to have had a marked influence on the actual amount of dose delivered to deep lung fibroblasts per WLM of exposure.

Summary

Studies have been conducted that demonstrate the usefulness of cellular damage as a biomarker of radiation dose to the lung. They suggest that initial cytogenetic damage in lung fibroblasts is a good measure of biological dose, but it is not related to the radiation or chemical sensitivity of animals for lung cancer induction. Similarly, the rate of loss of induced micronuclei from lung fibroblasts, which was used as a measure of tissue repair, does not appear to count for sensitivity for the induction of lung cancer among rodent species and strains, as expected,

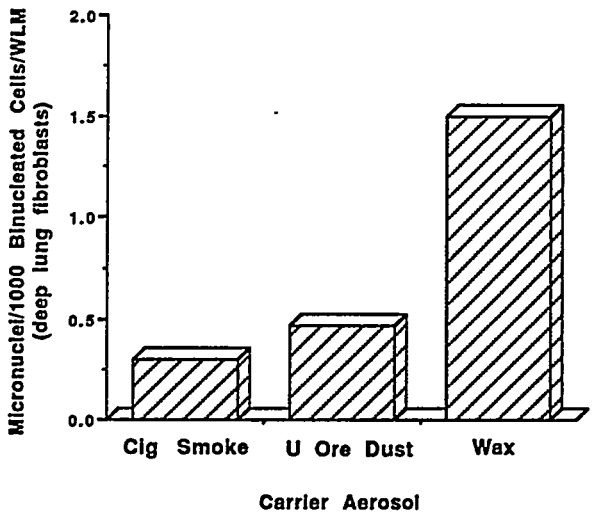

Figure 5. The Role of Aerosol Carriers (wax,uranium-ore dust, and cigarette-smoke particles) on the Induction of Micronuclei in the Deep Lung Following Inhalation of Radon and its Progeny

because removal of cells with potentially lethal damage may not affect cancer rates. In addition, an RBE of 65 was determined for low dose-rate radon-progeny exposure in vivo. This factor, combined with a rather high dose-rate effectiveness factor (6.0) for high vs. low dose-rate exposure, provide comparative risk estimations for cytogenetic damage. Finally, the influence of aerosol characteristics on dose and cytogenetic damage in deep lung fibroblasts was determined with 3 different aerosols. Further work is needed to characterize these aerosols, particularly in regard to the radon-progeny dose distribution in the respiratory tract. To make this research more useful for carcinoma risk evaluation, it is of *prime* importance to extend these studies to epithelial cells of the respiratory tract.

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Author Index

Author Index

Brooks, A.L., 23, 47, 53 57
Buschbom, R.L., 1, 19, 41
Cross, F.T., 41, 47, 53, 57
Dagle, G.E., 1, 41
Edmonds, C.G., 35
Eicher, E.M., 47
Fuciarelli, A.F., 35
Gies, R.A., 41, 47, 53
Gilbert, E.S., 1, 19
Groch, K.M., 23, 47, 57
Hill, R.L., 19
Hui, T.E., 53
Jostes, R.F., 23, 53, 57
Karagianes, M.T., 13
Khan, M.A., 47, 57
Ligotke, E.K., 13
Lutze, L.H., 53
Mann, D.B., 29
Miller, J.H., 35
Miller, R.C., 53
Murad, A.O., 29
Nelson, J.M., 53
O'Neill, J.P., 53
Park, J.F., 1
Petty, C.R., 41
Prather, J.C., 13
Saffer, J.D., 47
Sisk, E.C., 35
Smith, R.D., 35
Smith, L.G., 13
Smith, S.K., 13
Springer, D.L., 29, 35
Stillwell, L.C., 47, 53
Thrall, R.S., 47
Watson, C.R., 1, 13
Weller, R.E., 1
Wood, B., 23

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