exposed to plutonium. Furthermore, Dugan and Bedford (*17*) used low-passage normal primary diploid fibroblasts to measure induction of delayed chromosomal instability after irradiation and found no clear evidence for it. Observations of instabilities in normal human cells are therefore inconsistent; thus the question remains as to whether instability can occur in the cells of people exposed to radiation *in vivo*.

We previously examined the possibility of radiation-induced delayed chromosome aberrations in A-bomb survivors using the frequency of additional *de novo* translocations among clonally expanded T lymphocytes *in vivo* as an indicator (*18*). The results indicated that the frequency of new translocations among the clonal populations in A-bomb survivors was not higher than the frequency among non-clonal cells from controls, indicating a lack of instability *in vivo*. We concluded that radiation-induced chromosome instability is not as common as an increased frequency of translocations among clonally derived lymphocytes.

In the present work, the earlier study was extended by clonally expanding T cells from A-bomb survivors to determine whether chromosome instability can develop during long-term culture *in vitro*. Two proximally exposed A-bomb survivors were selected for the study because both were known to bear clonal chromosome aberrations with a high frequency (*18*). Since the frequencies of these clones were 10% and 50%, respectively, among the blood lymphocytes, the clonally derived cells must have proliferated extensively *in vivo*. Thus we anticipated that such clonal cells may exhibit significant induction of chromosome instability after long-term forcible cell proliferation *in vitro*. Multicolor FISH (mFISH) was used to detect newly arisen non-clonal chromosome aberrations that may occur in those clones. T-cell clonal populations from age- and sex-matched control individuals were also studied for comparison.

MATERIALS AND METHODS

Blood Donors

Peripheral blood lymphocytes were obtained from four female A-bomb survivors (cases 1–4). Table 1 gives information about the subjects. Briefly, cases 1 and 2 were survivors exposed to large doses of radiation (more than 1 Gy). Both were known to carry clonal chromosome aberrations with a high frequency (*18*). Cases 3 and 4 were survivors whose estimated doses were less than 5 mGy; they were selected as age- and sex-matched controls for cases 1 and 2, respectively. None of the donors had a medical history of cancer before blood was drawn.

These survivors belong to the Adult Health Study (AHS) cohort at the Radiation Effects Research Foundation (RERF) in Hiroshima (*19*). Their radiation doses were estimated using the Dosimetry System 2002 [DS02; ref. (*20*)]. A fixed weighting factor of 10 was used for neutrons to calculate the weighted total dose in Gy (*21*). This study is a part of our extensive research program for biological dose estimations for AHS individuals and was approved by the program review committee and the ethical review committee at RERF.

Lymphocyte Cloning and Culture

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque (LSM, Lymphocyte Separation Medium; MP Biomedicals, Aurora, OH) density gradient centrifugation. T lymphocytes were cloned and expanded *in vitro* by methods described elsewhere (*22*) with some modifications. PBMCs at a mean frequency of 0.5 cell/well were distributed into each well of a 96-well round-bottom plate (Corning, Corning, NY). GIT medium (Wako Pure Chemical Industry, Osaka, Japan) containing 9% fetal bovine serum (FBS; Intergen, New York, NY), 1% human AB-type serum, 2% L-glutamine (Invitrogen, Carlsbad, CA), 2% penicillin-streptomycin (Invitrogen), 1:6400 phytohemagglutinin (PHA; Difco Laboratories, Detroit, MI), and 10 ng/ml recombinant human interleukin 2 (rhIL-2; Pepro Tech, London, UK), was used. Feeder cells (5×10^4 allogeneic PBMCs and 10^4 lymphoblastoid cells, OKIB, irradiated with 50 and 100 Gy of X rays, respectively) were added. After about 2 weeks of incubation at 37°C in a humidified 5% CO_2/95% air incubator, each growing colony was transferred into one well of a 24-well plate (Corning) with the same culture medium containing 2.5×10^4 beads/ml of CD3/CD28 T-cell expander beads (Dynal Biotech ASA, Oslo, Norway) and cultured until several million cells were obtained. For cases 2, 3 and 4, the average culture time was approximately 4 weeks, and the number of population doublings was 23 to 25. In case 1, T-cell colonies that had been cloned previously (*22*) and cryopreserved were thawed and cultured in a 24-well plate under the same culture conditions.

mFISH Analysis

Chromosome slides were prepared by conventional air-drying methods 2 h after treatment of the cultured cells with colcemid (100 ng/ml) (*23*). mFISH was performed with SpectraVision DNA probes (Vysis, Downers Grove, IL) according to the manufacturer's protocol (*24*). The probes were denatured and hybridized to metaphase spreads at 37°C for two nights (~42 h). The slides were then washed in 0.4× SSC/0.3% NP-40 at 73°C for 2 min followed by a 2× SSC/0.1% NP-40 wash at room temperature for 1 min and finally a 2× SSC wash at room temperature for 2 min before subsequent application of a mounting medium (4′,6-diamidino-2-phenylindole, DAPI, 250 ng/ml) and a cover slip. Acquisition and analysis of mFISH images was performed using a CytoVision ChromoFluor System (Applied Imaging, Newcastle upon Tyne, UK). One hundred cells were scored for each clone.

Hematopoietic Colonies

As described elsewhere (*21*), approximately 500 CD34 (a hematopoietic stem cell surface marker) -positive cells were sorted from about 2 million PBMCs using a cell sorter (JSAN, Bay Bioscience, Kobe, Japan) and resuspended in 0.2 ml Iscove's MDM (Invitrogen) containing 2% FBS. The cell suspension was mixed with 2 ml methylcellulose medium (Methocult TM GF H4434V; Stemcell Technologies, Vancouver, Canada) containing recombinant human (rh) erythropoietin, rh stem cell factor, rhGM-CSF, rhG-CSF and rhIL-3 and then dispensed into two 35-mm petri dishes (about 1.1 ml each). After 10 days in culture at 37°C in 5% CO_2, the cells were treated with 100 ng/ml colcemid for 16 h and collected for chromosome analyses.

RESULTS

Characteristics of T-Cell Clones Obtained from the Peripheral Blood of A-Bomb Survivors

A total of 66 clones were established from two A-bomb survivors and two control individuals (see Table 1).

TABLE 1
Summary of the Study Subjects

Case no.	Sex	Age at the time of bombing (years)	Age when blood samples were collected (years)	DS02 bone marrow dose (mGy)	Blood sample used for the study	Information about *in vivo* clonal aberrations
1	F	20	65	1950	Frozen blood (16 years)[a]	Clone with t(4;6),t(5;13) in ~10% of the cells
2	F	13	72	1150	Freshly obtained blood	Clone with t(2;4) in ~50% of the cells
3	F	19	63	1.3	Frozen blood (17 years)[a]	No clone
4	F	13	70	1.7	Frozen blood (2 years)[a]	No clone

[a] Numbers in parentheses indicate the length of time the blood samples were kept in liquid nitrogen.

Case 1. This survivor had been exposed to a large dose of radiation (estimated dose is 1.95 Gy). Cells from this individual are known to bear an identical double translocation [t(4;6),t(5;13)] in about 10% of the lymphocytes (*18*). In the present experiments, 14 clones were studied that had been isolated and frozen 16 years ago (*22*). These consisted of four clones with a normal karyotype five with identical double translocations [t(4;6),t(5;13)] derived from the clonal cells *in vivo*, four with different structural aberrations, and one with mosaic X chromosome aneuploidy (45,X/46,XX) (Table 2).

Case 2. This person had been exposed to a large dose of radiation (estimated dose is 1.15 Gy). Cells from this person are also known to carry a clonal population of blood lymphocytes with high percentage of the clonal translocations, i.e., [t(2;4)] in about 50% of the cells (*18*). Twenty-two clones were obtained including eight clones with normal karyotypes, eight with the clonal translocations [t(2;4)], five with other structural aberrations, and one with a chromosome 2 trisomy (Table 3).

Case 3. This donor was selected as an age- and sex-matched control for Case 1. Of the 12 clonal cell populations obtained, 10 clones had a normal karyotype, one had a deletion of chromosome 5q, and one was mosaic for X chromosome aneuploidy (45,X/47,XXX) (Table 4).

Case 4. This donor was selected as an age- and sex-matched control for Case 2. Eighteen clones were obtained, of which 14 clones had a normal karyotype, two had a monosomy for the X chromosome, one had a chromosome 22 trisomy, and one had a mosaic X chromosome aneuploidy (45,X/46,XX/47,XXX) (Table 5).

Origin of Clonal Chromosome Aberrations In Vivo

The origin of the clonal chromosome aberration in case 1 [t(4;6),t(5;13)] was previously confirmed as deriving from a single bone marrow stem cell, because identical double translocations were observed not only in peripheral blood lymphocytes (both T and B) but also in stem cell-derived BFU-E colonies (*22*). In case 2, the identical translocations [t(2;4)] were detected in both CD4 and CD8 T-lymphocyte populations (*25*), but no further examination had been done. In the present study, CD34-positive cells were cultured from blood mononuclear cells, and a t(2;4) translocation was found in nearly 80% of the metaphases examined (156/200) with the FISH method

TABLE 2
Frequency of Additional Chromosome Aberrations among Clonally Expanded T Lymphocytes *In Vitro* in Case 1

			Additional chromosome aberrations[a]								
			Structural aberrations							Aneuploidy	
Clone no.	Karyotype	No. of cells examined	t	der	dic	dup	del	f	total	gain	loss
1-1	46,XX	100		1		1	3	1	6		8
1-2	46,XX	100	1				1	2	4	1	3
1-3	46,XX	100					1	6	7		2
1-4	46,XX	100						1	1		3
1-5	45,X/46,XX	86/14				1	3		4	1	5
1-6	t(4;6),t(5;13)	100	3	1		5 (4)	21 (9)	4	21	6 (4)[b]	10
1-7	t(4;6),t(5;13)	100		3				3	6		3
1-8	t(4;6),t(5;13)	100			1		2	2	5	3	3
1-9	t(4;6),t(5;13)	100				1	1		2	1	2
1-10	t(4;6),t(5;13)	100		3 (2)					2		3
1-11	t(X;2),t(5;12),t(6;11;10)	100	1			3 (2)	2		5	3	7
1-12	t(7;12)	100					2	1	3		5
1-13	t(3;21)	100			1			1	2	1	3
1-14	t(3;6;12)	100					1	2	3	3	5
	Total	1400	5	7	2	9	25	23	71	17	62

Note. t = translocation, der = derivative chromosome, dic = dicentric chromosome, dup = duplication, del = deletion, f = fragment, gain = gain of chromosome, loss = loss of chromosome.

[a] Number in parentheses indicates the number of events (see the Results for details).

[b] An extra identical aberration [+del(1)(p11)] was detected in three cells.

TABLE 3
Frequency of Additional Chromosome Aberrations among Clonally Expanded T Lymphocytes *In Vitro* in Case 2

Clone no.	Karyotype	No. of cells examined	Additional chromosome aberrations								
			Structural aberrations							Aneuploidy	
			t	der	dic	dup	del	f	total	gain	loss
2-1	46,XX	100		2			2	3	7	4	9
2-2	46,XX	100	1				1		2	1	7
2-3	46,XX	100		1					1	3	3
2-4	46,XX	100						2	2	2	8
2-5	46,XX	100	1				2	1	4	3	14
2-6	46,XX	100					3	4	7	1	9
2-7	46,XX	100	1				6	2	9	1	6
2-8	46,XX	100	3	1			3		7		3
2-9	47,XX,+2	100	1	1			4		6	1	10
2-10	46,XX,t(2;4)	100	1			7 (1)	1	2	5	4	8
2-11	46,XX,t(2;4)	100					2	1	3	2	1
2-12	46,XX,t(2;4)	100	1				5		6	2	10
2-13	46,XX,t(2;4)	100	1				2	7	10	4	4
2-14	46,XX,t(2;4)	100	1		1		2		4	1	3
2-15	46,XX,t(2;4)	100	1	1		1	4	1	8	2	10
2-16	46,XX,t(2;4)	100		9 (2)			1	1	4		3
2-17	46,XX,t(2;4)	84					1		1	4 (1)[a]	6
	46,XX,t(3;22), t(4;8)	16							0		
2-18	46,XX,t(1;10)	100	1	1			3	1	6	3	11
2-19	46,XX,t(1;12)	100	2		1		1	1	5	1	3
2-20	47,XXX,t(13;18;20)	100		3	18		2	1	24	6	2
2-21	45,X, t(2;8;17)	100					2		2	2	9
2-22	45,X,t(2;16)	100					1	1	2	1	3
	Total	2200	15	12	20	2	48	28	125	45	142

Note. Abbreviations are defined in Table 2.
[a] Extra derivative chromosome [+der(2)] that is the counterpart of an *in vivo* clonal translocation was detected in 4 cells.

using probes specific for chromosomes 2 (Green) and 4 (Red). We thus concluded that the t(2;4) clone in case 2 derived from a hematopoietic stem cell.

Additional Chromosome Aberrations Detected in Clonally Expanded T Lymphocytes

Data for individual clones from each blood donor are shown in Tables 2–5. Various types of new "additional" aberrations were detected among the 100 cells from the clones examined. Further, identical aberrations were frequently encountered in multiple cells in the population of clonal cells. Since these aberrations almost certainly arose only once during clonal culture *in vitro*, each identical aberration was counted as a single event. The numbers in parentheses in Tables 2–5 indicate the number of such events (intraclonal clones or subclonal clones).

TABLE 4
Frequency of Additional Chromosome Aberrations among Clonally Expanded T Lymphocytes *In Vitro* in Case 3

Clone no.	Karyotype	No. of cells examined	Additional chromosome aberrations								
			Structural aberrations							Aneuploidy	
			t	der	dic	dup	del	f	total	gain	loss
3-1	46,XX	100					2	2	4		4
3-2	46,XX	100					1	1	2	2	4
3-3	46,XX	100				1	2		3	2	4
3-4	46,XX	100	1	3			1		5	3	4
3-5	46,XX	100	1	1			2	4	8		8
3-6	46,XX	100							0		3
3-7	46,XX	100				1	3	4	8	1	4
3-8	46,XX	100				1	1	3	5		4
3-9	46,XX	100	1	7 (4)				1	6	2	1
3-10	46,XX	100							0	1	5
3-11	45,X/47,XXX	35/65							0		3
3-12	46,XX,del(5q)	100					2		2	5	4
	Total	1200	3	8	0	3	14	15	43	16	48

Note. Abbreviations are defined in Table 2.

TABLE 5
Frequency of Additional Chromosome Aberrations among Clonally Expanded T Lymphocytes *In Vitro* in Case 4

Clone no.	Karyotype	No. of cells examined	Additional chromosome aberrations								
			Structural aberrations							Aneuploidy	
			t	der	dic	dup	del	f	total	gain	loss
4-1	46,XX	100					5	6	11	3	3
4-2	46,XX	100		1		1	2	2	6	2	6
4-3	46,XX	100		1			1		2	1	5
4-4	46,XX	100					3		3	3	5
4-5	46,XX	100					2		2		10
4-6	46,XX	100					11 (6)	3	9	4	1
4-7	46,XX	100					1	1	2		5
4-8	46,XX	100						2	2		6
4-9	46,XX	100	1						1	3	2
4-10	46,XX	100					2	3	5	1	4
4-11	46,XX	100					1		1	1	4
4-12	46,XX	100					1	2	3	5	4
4-13	46,XX	100					3	1	4	1	4
4-14	46,XX	100	1		1		10 (5)	1	8	5	5
4-15	45,X	100					3	2	5	2	7
4-16	45,X	100	2		1	2	3 (2)	2	9	1	6
4-17	47,XX,+22	100					2		2	3	1
4-18	45,X/ 46,XX /47,XXX	80/3/17					1		1		8
	Total	1800	4	2	2	3	40	25	76	35	86

Note. Abbreviations are defined in Table 2.

Most of the clonal populations we observed appeared to be of single cell origin except for four clones; clone no. 2-17 appears to have started with two cells instead of one. The other three clones (nos. 1-5, 3-11, 4-18) consisted of cells with X-chromosome aneuploidy. Although it is not possible to determine whether they were derived from multiple cells or were the result of sex-chromosome loss or non-disjunction during clonal expansion, the latter possibility appears to be more likely since X-chromosome aneuploidy is known to take place commonly in cultured lymphocytes from elderly women (*26*). The above three X-chromosome aneuploid clones, together with two other clones, that showed autosomal trisomy (nos. 2-9, 4-17) were categorized as a "normal karyotype" in Table 6.

Statistical Analyses of the Additional Chromosome Aberration Frequencies in Exposed and Control Subjects

1. Reciprocal translocations (t)

It is generally thought that since reciprocal translocations do not gain or lose DNA, they are not subject to negative selection that would result in their reduced fraction during extensive cell divisions (*27*). Therefore, if reciprocal translocations had been induced by genomic instability they must have accumulated among descendant cells during *in vitro* culture of the clones. The frequency of newly induced additional reciprocal translocations (t) in the exposed subjects (cases 1 and 2) was 0.56% (20/3600), whereas it was 0.23% (7/3000) in the controls (cases 3 and 4) (Table 6). This difference is not statistically significant ($P = 0.070$) with the Wald test using the quasi-likelihood method. This same statistical evaluation was applied in the following section.

2. Derivative chromosome (der)

An example of derivative chromosomes is shown in Fig. 1b. These chromosomes are composed of non-reciprocal translocations and hence are accompanied by gains and losses of DNA segments that may confer a growth disadvantage to the cells. They seem to arise as a result of chromatid-type exchanges in S phase. The frequency of derivative chromosomes was 0.53% (19/3600) in the exposed cases, and 0.33% (10/3000) in the controls (Table 6). The difference was not statistically significant ($P = 0.371$).

mFISH is recognized as the best technique available for the detection of interchromosomal exchanges such as translocations and derivative chromosomes described here. Since both translocations and derivative chromosomes are stable-type aberrations and thus can be transferred to the next generation, the combined data for (t + der) were also analyzed. The results indicated no statistically significant difference between the two groups ($P = 0.11$).

3. Dicentric chromosomes (dic)

Dicentric chromosomes, or dicentrics, are representative of unstable-type chromosome aberrations that are easily detected but are lost over time during successive cell divisions due to problems associated with mitotic chromosome segregation. Consequently, it is generally thought that such aberrations do not accumulate in a cell population. As shown in Tables 2–5, the dicentric frequencies observed in the subjects were 0.1% (2/1400, case 1), 0.9% (20/2200, case 2), 0% (0/1200, case 3), and 0.1% (2/1800, case 4), respectively. Three cases (1, 3, 4) had

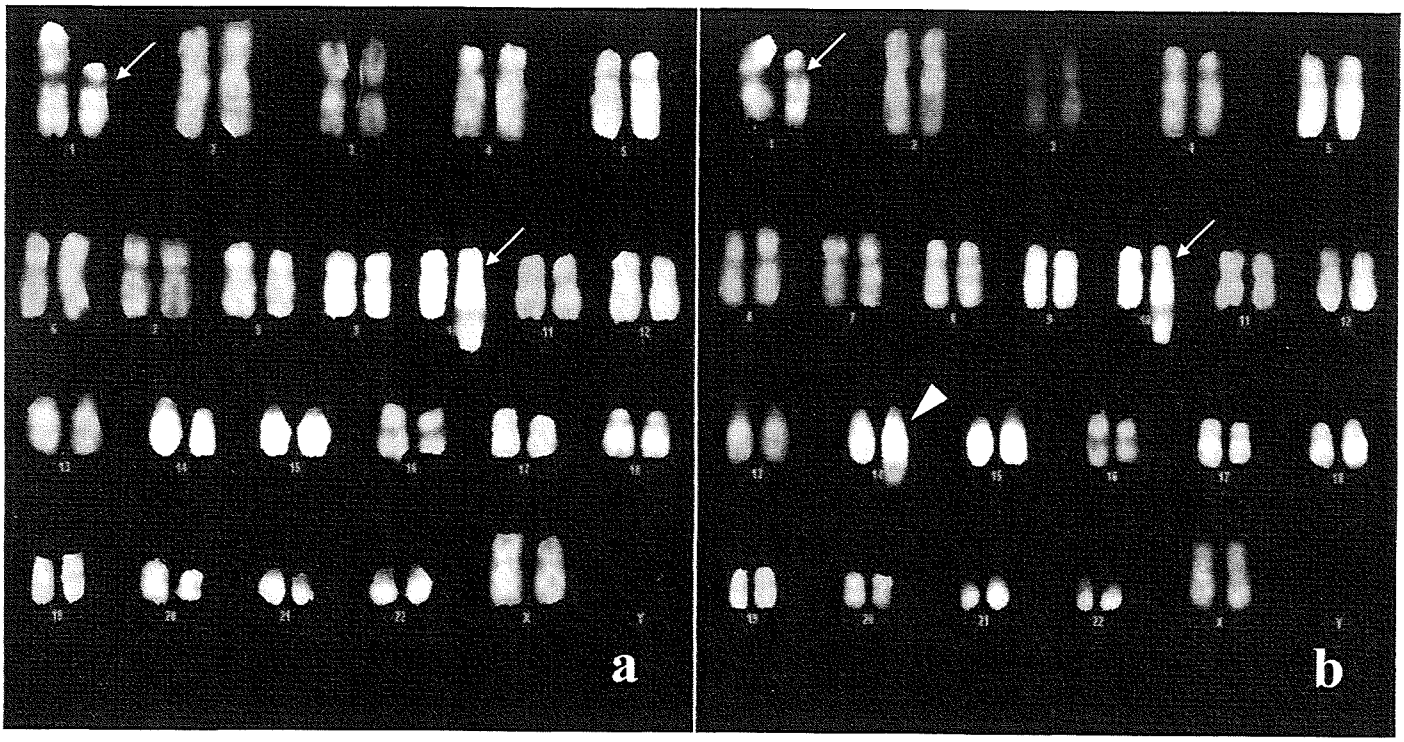

FIG. 1. Detection of additional chromosome aberrations with mFISH (Clone 2-18 in Table 3). Panel a: Translocation alone, 46,XX,t(1;10). Panel b: Translocation plus non-clonal aberration, 46,XX,t(1;10),der(14)t(2;14). Arrows indicate the translocation between chromosomes 1 and 10 and the arrowhead shows the additional aberration (derivative chromosome) involving chromosomes 2 and 14. Two normal intact number 2 chromosomes are evident.

relatively low levels of dicentric frequencies corresponding to control values when human blood lymphocytes were examined after short-term culture (0.1–0.2%) (*28*). However, clone 2-20 from case 2 developed a relatively high frequency of apparent dicentric chromosomes in the culture (18/100, Table 2). None, however, had accompanying fragments, which indicates that they resulted from the terminal fusion of two chromosomes (see Discussion). Six dicentrics were detected independently in other clones, some with fragments, and were considered to be true dicentrics since their breakpoints were distributed in non-terminal regions of the chromosomes. When clone 2-20 was excluded from the analysis, the dicentric frequency in case 2 was similar to those seen for samples from non-exposed controls (0.1%, 2/2100) (*28*).

4. Other aberrations (deletions, duplications, fragments and aneuploidy)

Although the mFISH technique is not highly sensitive for detecting structural chromosome aberrations such as duplications, deletions and fragments (i.e., without color change), some of those aberrations were clearly detectable with mFISH in combination with DAPI counterstaining. The numbers of such aberrations detected in this study are summarized in Table 6. The pooled frequencies of these three types of aberrations (dup + del + f) was 3.75% (135/3600) in the exposed cases and 3.33% (100/3000) in the controls. The difference between the two groups was not statistically significant ($P > 0.5$).

On the other hand, mFISH is the most reliable method for detecting aneuploidy, i.e. chromosome gains or losses in metaphase spreads. However, there was no statistically significant difference in the observed frequency of chromosome gain between the exposed (1.7%, 62/3600) and the control groups (1.7%, 51/3000) ($P > 0.5$). The observed frequency of chromosome loss was slightly higher in the exposed group (5.67%, 204/3600) than in the control group (4.47%, 134/3000), but again the difference was not statistically significant ($P = 0.069$). As shown in Table 6, the occurrence of chromosome loss was three times higher ($n = 338$) than that of chromosome gain ($n = 113$), which could indicate that some of the losses were due to artifacts that can occur during preparation of metaphase spreads (*29*).

5. All structural chromosome aberrations

The combined data for all structural chromosome aberrations of the exposed and the control groups were compared, and no statistically significant difference was observed ($P = 0.142$).

DISCUSSION

To investigate radiation-induced genomic instability in human peripheral blood lymphocytes, T lymphocytes from A-bomb survivors were clonally expanded *in vitro*, and the frequencies of additional *de novo* chromosome aberrations arising in individual clones during the

TABLE 6
Frequency of Karyotypes and Additional Chromosome Aberrations in T Cell *In Vitro* Clones from Exposed and Control Subjects as well as among Different Karyotypes

Karyotype of the clone[a]	No. of clones	Total cells	Additional chromosome aberrations		
			Structural aberrations		
			t	der	dic
Exposed (cases 1 + 2)					
Normal	14	1400	8 (0.57%)	6 (0.43%)	0 (0.00%)
In vivo-derived clonal aberration	13	1300	8 (0.62%)	9 (0.69%)	2 (0.15%)
Other structural aberrations	9	900	4 (0.44%)	4 (0.44%)	20 (2.22%)
Total[b]	36	3600	20 (0.56%)	19 (0.53%)	22 (0.61%)
Control (case 3 + 4)					
Normal	29	2900	7 (0.24%)	10 (0.34%)	2 (0.07%)
Other structural aberrations	1	100	0 (0.00%)	0 (0.00%)	0 (0.00%)
Total[b]	30	3000	7 (0.23%)	10 (0.33%)	2 (0.07%)

[a] Clones with aneuploid karyotypes were classified as "Normal". t = translocation, der = derivative chromosome, dic = dicentric chromosome, dup = duplication, del = deletion, f = fragment, gain = gain of chromosome, loss = loss of chromosome.
[b] Numbers in total indicate the number of events.

cultivation period were determined with mFISH. The results did not provide any conclusive evidence for the presence of chromosome instability, although the observed frequency estimate was slightly higher in the exposed cases. The results are therefore in accord with our previous study in which no increased levels of chromosome instability were found in clonally expanded lymphocyte populations *in vivo* in A-bomb survivors (*18*). Our previous *in vivo* and present *in vitro* observations will raise a question about the general involvement of chromosome instability as an initiating step in radiation-induced carcinogenesis.

In Vivo Clonal Chromosome Aberrations

As described in the Results section, the clonal chromosome aberrations observed in a large number of cells from case 1 and case 2 were both confirmed as being derived from aberrant bone marrow stem cells. It was previously found that these clones were induced after exposure to A-bomb radiation (*18*); thus the stem cells and their progenitors must have divided extensively. Nonetheless, the spontaneous frequency of new aberrations among the clonal cell populations was not much different from that in the control individuals (P = 0.114 for t + der and P = 0.13 for total structural aberrations). This may indicate that age-related increases in the frequency of chromosome aberrations in blood lymphocytes may not be due to an increased number of cell divisions of the stem/progenitor cells but rather to the systemic physiological conditions in the host (*18*).

Derivative Chromosomes

With the mFISH technique, it was possible to accurately identify chromosome aberrations such as derivative chromosomes (der) and duplications (dup) as additional aberrations in metaphase. Such aberrations are unusual in short-term cultures of lymphocytes (i.e., 2–3 days), which are used for standard human chromosome tests. In particular, derivative chromosomes are observed only rarely in normal cells, partly because their correct identification is very difficult with conventional cytogenetic techniques other than mFISH. In the present study, derivative chromosomes could be observed clearly in both exposed and control subjects, and there was no statistically significant difference in frequency between the two groups (P = 0.371). Therefore, it appears likely that most of these rare aberrations occurred during the long-term culture of the lymphocytes regardless of previous radiation exposure. In contrast to our results, Holmberg *et al.* described the appearance of derivative (or marker) chromosomes in human lymphocytes that were irradiated with X rays and subjected to long-term culture *in vitro* (*7*). This discrepancy might be attributed to either the single blood donor used for the Holmberg study and/or possibly suboptimal culture conditions for T lymphocytes because the rhIL2 used in the present study was not available in the 1980s when the Holmberg study was conducted.

One Apparently Unstable Clone with Possible Telomere Shortening

In clone no. 2-20 (case 2), 18 dicentric chromosomes were observed, and none of them contained acentric fragments. DAPI banding (counterstaining for mFISH) indicated that most of these dicentrics appeared to be end-to-end fusions of whole chromosomes. Further FISH analysis with pantelomere probes showed one telomere signal at each point of chromatid fusion (data not shown), suggesting a telomere-telomere fusion as a result of telomere shortening in at least some of the

TABLE 6
Extended

Additional chromosome aberrations					
Structural aberrations				Aneuploidy	
dup	del	f	total	gain	loss
2 (0.14%)	29 (2.07%)	22 (1.57%)	67 (4.79%)	18 (1.29%)	90 (6.43%)
7 (0.54%)	30 (2.31%)	21 (1.62%)	77 (5.92%)	24 (1.85%)	66 (5.08%)
2 (0.22%)	14 (1.56%)	8 (0.89%)	52 (5.78%)	20 (2.22%)	48 (5.33%)
11 (0.31%)	73 (2.03%)	51 (1.42%)	196 (5.44%)	62 (1.72%)	204(5.67%)
6 (0.21%)	52 (1.79%)	40 (1.38%)	117 (4.03%)	46 (1.59%)	130(4.48%)
0 (0.00%)	2 (2.00%)	0 (0.00%)	2 (2.00%)	5 (5.00%)	4 (4.00%)
6 (0.20%)	54 (1.80%)	40 (1.33%)	119 (3.97%)	51 (1.70%)	134(4.47%)

chromosomes in this clonal cell population. It has been reported in several studies that such telomere dysfunction was one of the signs of delayed chromosomal instability after irradiation (*10, 12, 30–32*). Alternatively, because telomere length in human lymphocytes decreases with increased age *in vivo* (*33, 34*), the observed end-to-end chromosome fusions in clone 2-20 may be due to the advanced age of the donor. Further investigations in lymphocytes with younger donors will be necessary to test this hypothesis.

It was reported decades ago that many dicentrics without acentric fragments were found in senescent human embryonic fibroblasts, and they were confirmed to be end-to-end fusions of whole chromosomes after banding analysis (*35*). It was asserted that such aberrations were senescence-related chromosome changes and thus were different from radiation-induced genetic instability (*17*). In addition to the present study, many apparently similar dicentric chromosomes were detected in a T-lymphocyte clone from one healthy individual in the control group (Kodama *et al.*, unpublished data). After banding analysis, it was found that these dicentrics were due to terminal fusions between the two chromosomes. Therefore, under the present conditions, we concluded that the observed apparent dicentric chromosomes in one exposed subject do not provide indisputable evidence for chromosomal instability after radiation exposure.

Possible Reasons for a Lack of Instability

Although a number of studies have used human cells and focused on chromosome aberrations as an index for radiation-induced genomic instability, the results, both positive (*7–12*) and negative (*15–17, 36*), have not always been concordant. Such inconsistencies might be due partly to different genetic backgrounds among individuals (*8, 9, 13, 14*). On the other hand, inconsistencies in these results might be attributed to the different circumstances in which the cells survived (i.e., *in vivo* or *in vitro*). Wright and Coates (*5*) pointed out that the occurrence of radiation-induced chromosome instability *in vivo* is much less than that *in vitro* and suggested the presence of some cellular defense mechanisms *in vivo* that could efficiently eliminate aberrant cells from tissues. Large differences in the number of cell divisions between *in vitro* and *in vivo* test systems may also affect these results (*8*). The T-cell cloning techniques used in this study required long-term forcible cell proliferation and thus might increase opportunities to obtain new *de novo* chromosome aberrations in culture.

It is possible that individuals among the A-bomb survivors who were genetically predisposed to the induction of radiation-associated instability were already eliminated from the population due to early death from cancer. However, this seems unlikely because cancer risk started to increase years after the radiation exposure and is still elevated today (*37*).

In the present study, an effort was made to find radiation-induced chromosome instability in clonally cultured T lymphocytes from A-bomb survivors, but no clear evidence for the presence of instability was found. The results are interpreted to indicate that the instability, if it exists, is not an event that is frequent enough to be easily detected in the experiments of the size we conducted, which involved the examination of more than 5000 cells with the mFISH method.

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Original Contribution

Positive Associations Between Ionizing Radiation and Lymphoma Mortality Among Men

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The authors investigated the relation between ionizing radiation and lymphoma mortality in 2 cohorts: 1) 20,940 men in the Life Span Study, a study of Japanese atomic bomb survivors who were aged 15–64 years at the time of the bombings of Hiroshima and Nagasaki, and 2) 15,264 male nuclear weapons workers who were hired at the Savannah River Site in South Carolina between 1950 and 1986. Radiation dose-mortality trends were evaluated for all malignant lymphomas and for non-Hodgkin's lymphoma. Positive associations between lymphoma mortality and radiation dose under a 5-year lag assumption were observed in both cohorts (excess relative rates per sievert were 0.79 (90% confidence interval: 0.10, 1.88) and 6.99 (90% confidence interval: 0.96, 18.39), respectively). Exclusion of deaths due to Hodgkin's disease led to small changes in the estimates of association. In each cohort, evidence of a dose-response association was primarily observed more than 35 years after irradiation. These findings suggest a protracted induction and latency period for radiation-induced lymphoma mortality.

lymphoma; mortality; nuclear weapons; radiation, ionizing

Abbreviations: CI, confidence interval; ERR, excess relative rate; ICD, *International Classification of Diseases*; LRT, likelihood ratio test; LSS, Life Span Study; ND, not determined; NHL, non-Hodgkin's lymphoma; SRS, Savannah River Site.

Ionizing radiation has been considered as a cause of lymphoma by a number of investigators. In a review of this literature, Boice (1) concluded that the evidence of association between ionizing radiation and non-Hodgkin's lymphoma (NHL) is extremely weak and that there is no evidence of association between radiation and Hodgkin's disease. The United Nations Scientific Committee on the Effects of Atomic Radiation noted that studies of NHL following external exposure to ionizing radiation have yielded mixed results and concluded that overall there is little evidence of an association between NHL and external exposure to ionizing radiation (2). Ron (3) reached a similar conclusion, noting that evidence of association between radiation and NHL has been inconsistent and Hodgkin's disease has rarely been related to radiation exposure; and Melbye and Trichopoulos (4) stated that there is no evidence that ionizing radiation causes NHL. However, this conclusion is not universally shared. Hartge et al. argued that the evidence suggests that ionizing radiation probably causes lymphoma (5) and observed that high doses of ionizing radiation appear to be associated with lymphoma risk in some studies of radiotherapy (6).

Lack of a consistent association between ionizing radiation and lymphoma could mean that there is no causal relation or that a causal relation is obscured by bias or deficiencies in exposure measurement, case classification, duration of follow-up, or some combination of these factors. Given that lymphoma is often an indolent disease, long-term studies of radiation-exposed populations may be needed to observe an effect. The development of nuclear weapons in the early 1940s led to 2 types of epidemiologic studies that can now provide evidence regarding the radiation-lymphoma association: studies based on follow-up of workers exposed to ionizing radiation during nuclear weapons

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production and studies based on follow-up of people exposed to ionizing radiation from the use of nuclear weapons. Most prominent among the latter is the Life Span Study (LSS), a study of Japanese survivors of the atomic bombings of Hiroshima and Nagasaki. Radiation risk estimates from studies of nuclear workers are often compared with estimates from the LSS in order to evaluate the consistency of risk estimates in a population that includes people exposed to acute high doses with estimates from populations that are chronically exposed to low doses (7–9).

We examined the association between ionizing radiation and lymphoma mortality in a US occupational cohort and in a sample of LSS atomic bomb survivors and compared findings from the 2 populations. Follow-up of each cohort commenced in 1950 and spanned approximately 5 decades. To the extent possible, we conducted these analyses as parallel analyses employing comparable methods. We focused, in particular, on variation in the associations between radiation dose and lymphoma mortality by time since exposure.

MATERIALS AND METHODS

The LSS cohort includes 86,611 people who were alive at the time of the 1950 Japanese census, reported being in Hiroshima or Nagasaki at the time of the bombings (August 1945), and had dose estimates based on the DS02 dosimetry system (10). Follow-up for ascertainment of vital status and cause-of-death information started on October 1, 1950, and continued until December 31, 2000.

The Savannah River Site (SRS) was constructed near Aiken, South Carolina, in 1950 as a facility to produce materials for the US nuclear weapons program. A cohort of 18,883 workers who were hired at the SRS prior to 1987, who worked there for at least 90 days, who were not known to have been employed at another US Department of Energy facility, and who had complete information on name, Social Security number, sex, date of birth, and date of hire was enumerated (11). Vital status and cause-of-death information were ascertained through December 31, 2002.

Cohort restrictions for comparability

Since over 95% of the collective dose at SRS was incurred by males, there was little ability to estimate risk due to radiation exposure among female SRS workers. We therefore restricted the analyses to males in both cohorts. Since the youngest age at hire at SRS was 15 years and most SRS workers terminated their employment by age 65 years, LSS analyses were restricted to people who were aged 15–64 years at the time of the bombings. This resulted in a cohort of 15,264 male SRS workers and a cohort of 20,940 male LSS subjects who were aged 15–64 years at the time of the bombings.

Dosimetry data

For the LSS, we used DS02 revised colon dose estimates adjusted for dosimetry errors, with shielded kerma estimates above 4 Gy truncated to 4 Gy (12). For consistency with analyses of the SRS cohort, dose estimates calculated as the sum of the γ-radiation dose plus 10 times the neutron dose are expressed in sieverts; some recent reports on LSS analyses refer to this quantity as the weighted dose in grays (13, 14). Interactions between radiation and lymphocytes may occur in the lymphatic or circulatory system at a variety of anatomic sites; the choice of target organ for dose estimation may depend on the characteristics of the lymphoma, including anatomic location (15, 16). The colon dose has been taken as a representative dose to the organs involved at a variety of anatomic locations, similar to the approach employed in prior analyses of solid cancers (17). The colon dose estimate has been used by previous investigators as an estimate comparable to the quantity estimated by the radiation dosimeters worn by nuclear industry workers (i.e., the "deep dose").

For SRS workers, the exposure of interest was defined as cumulative whole-body radiation dose equivalent from external sources and tritium received during employment at the site, expressed in sieverts; neutron doses were multiplied by a factor of 10. Personal radiation monitoring data were available for the period 1950–1999. Whole-body doses were estimated for work-years with missing dose data using dose estimates from adjacent time periods and average values for similar workers; estimated annual doses constituted 4% of employment years for male workers (18).

Outcome definitions

In the LSS, underlying cause of death was coded according to the *International Classification of Diseases*, Ninth Revision (ICD-9), which was issued in 1977. In the SRS study, underlying cause of death was coded according to the Eighth Revision of the ICD (ICD-8) for deaths occurring prior to 1979 and according to the ICD revision in effect at the time of death for deaths occurring in 1979 or later. (The Tenth Revision of the ICD (ICD-10) was issued in 1992.)

As in prior analyses (17, 19), we examined the broad category of malignant lymphoma (ICD-8 and ICD-9 codes 200–202; ICD-10 codes C81–C85). In addition, we examined the subcategory of NHL (ICD-8 and ICD-9 codes 200 and 202; ICD-10 codes C82–C85). There were too few deaths due to Hodgkin's disease to support separate analyses of that outcome in these cohorts.

Statistical methods

Poisson regression methods were used. The analytical data file for the LSS cohort consisted of a tabulation of person-time and numbers of deaths by city, age at exposure (in 5-year intervals), attained age (in 5-year intervals), calendar time (1950–1952, 1953–1955, and then 5-year intervals up to 1995, 1996–1997, and 1998–2000), and dose (<0.005, 0.005–<0.02, 0.02–<0.04, 0.04–<0.06, 0.06–<0.08, 0.08–<0.1, 0.1–<0.125, 0.125–<0.150, 0.150–<0.175, 0.175–<0.2, 0.2–<0.25, 0.25–<0.3, 0.3–<0.5, 0.5–<0.75, 0.75–<1, 1–<1.25, 1.25–<1.5, 1.5–<1.75, 1.75–<2, 2–<2.5, 2.5–<3, and ≥3 Sv). The analytical data

file for the SRS cohort consisted of a tabulation of person-time and events by attained age (in 5-year intervals), race (black vs. other), year of birth (before 1915, 1915–1924, 1925–1929, 1930–1934, 1935–1949, or 1950 or later), pay code (paid monthly, weekly, or hourly), employment status (employed, terminated within the last 2 years, or terminated more than 2 years prior, classified separately for risk ages <62 years and ≥62 years) (20–22), and dose (0, >0–<0.005, 0.005–<0.02, 0.02–<0.04, 0.04–<0.06, 0.06–<0.08, 0.08–<0.1, 0.1–<0.125, 0.125–<0.150, 0.150–<0.175, 0.175–<0.2, 0.2–<0.25, 0.25–<0.3, and ≥0.3 Sv).

Covariate control was achieved through background stratification of regression models. In analyses of the LSS cohort, the stratifying factors were attained age, age at exposure, and city; in analyses of the SRS cohort, the stratifying factors were attained age, birth cohort, race, pay code, and employment status. Radiation dose-mortality associations were estimated via a regression model of the form

$$\text{rate} = e^{\alpha_i}(1 + \beta x),$$

where α_i indexes the stratum-specific mortality rate in the absence of radiation exposure and $\hat{\beta}$ provides an estimate of the excess relative rate (ERR) per sievert (23, 24).

In analyses of the LSS cohort, x represents the estimated radiation dose delivered at the time of the bombings in August 1945. Since follow-up of the LSS cohort began in October 1950, this implies a minimal lag of approximately 5 years between exposure and its effect. We also present results from analyses in which we assumed that there was no excess risk during the period 1950–1955; that is, a minimum latency period of approximately 10 years was assumed. A 10-year lag assumption has been used in previous nuclear worker studies that examined lymphoma mortality (25, 26). We refer to analyses of LSS data that examine excess mortality risk since 1950 and since 1956 as analyses carried out under 5- and 10-year lag assumptions, respectively. In analyses of the SRS cohort, x represents the cumulative radiation dose under a 5- or 10-year lag assumption. Lagging dose assignment by L years means that an increment of dose was included in the calculation of cumulative dose at time t if it had been received at or before time $t - L$ years; person-time and events at time t were then classified according to that category of lagged cumulative dose.

The dose range in the LSS, 0–4 Sv, was wider than the dose range in the SRS study (0–<0.5 Sv). In order to evaluate dose-response associations over a comparable range of doses, we also conducted analyses based upon LSS data limited to the 19,183 survivors with doses in the range of 0–<0.5 Sv.

In analyses of the LSS cohort, we assessed variation in radiation risk with time since exposure via a regression model of the form

$$\text{rate} = e^{\alpha_i}(1 + \beta_1 x\text{Period1} + \beta_2 x\text{Period2} + \beta_3 x\text{Period3} + \beta_4 x\text{Period4}),$$

where Period1–Period4 are indicator variables for the calendar time periods 1950–1970, 1971–1980, 1981–1990, and 1991–2000, respectively. The values $\hat{\beta}_1, \hat{\beta}_2, \hat{\beta}_3$, and $\hat{\beta}_4$ provide estimates of the ERR per 1-Sv dose during the periods 5–25, 26–35, 36–45, and 46–55 years after the bombings. In analyses of the SRS cohort, we fitted a model of the form

$$\text{rate} = e^{\delta_i}(1 + \phi_1 d_1 + \phi_2 d_2 + \phi_3 d_3),$$

where d_1–d_3 represent the cumulative radiation doses accrued in the exposure time windows 5–25, 26–35, and ≥36 years prior to observation of a person-year or event and $\hat{\phi}_1, \hat{\phi}_2$, and $\hat{\phi}_3$ provide associated estimates of the ERR per 1-Sv dose.

We estimated parameters using the EPICURE statistical package (Hirosoft International Corporation, Seattle, Washington); for consistency with recent reports (2, 26), we generated 90% confidence intervals for estimated parameters via the likelihood method (27). In some analyses, confidence bounds could not be determined (designated "not determined" (ND)). In order to aid interpretation of model fittings, we report the 1-sided P value derived via a likelihood ratio test (LRT) for each reported point estimate. Tabulations of observed versus expected numbers of deaths by category of cumulative dose are reported; we calculated expected counts for each cell of the person-time table using a regression model that included all variables except the dose term.

RESULTS

With follow-up through 2000, 90 malignant lymphoma deaths were observed among the male atomic bomb survivors exposed at ages 15–64 years, including 6 deaths from Hodgkin's disease (Table 1). Sixty-three malignant lymphoma deaths occurred among residents of Hiroshima (58 due to NHL) and 27 malignant lymphoma deaths occurred among residents of Nagasaki (26 due to NHL). No deaths due to malignant lymphoma occurred among survivors at attained ages less than 30 years. In the SRS cohort, 56 lymphoma deaths were observed; 5 of these deaths were due to Hodgkin's disease. One death due to malignant lymphoma was observed among black males (it was a case of NHL), and 18, 14, and 24 deaths due to malignant lymphoma were observed among workers paid monthly, weekly, and hourly, respectively. Three deaths due to malignant lymphoma occurred among actively employed SRS workers (all were cases of NHL) and 6 deaths occurred within 2 years of termination of employment (all were cases of NHL), while the remaining 47 deaths due to malignant lymphoma occurred 2 or more years after termination of employment at SRS (42 due to NHL).

In the LSS, the estimated ERR of malignant lymphoma per sievert, under a 5-year lag assumption, was 0.79 (90% confidence interval (CI): 0.10, 1.88). The goodness of model fit was slightly improved, and the magnitude of association was slightly increased, upon exclusion of deaths due to Hodgkin's disease (Table 2). Under a 10-year lag assumption, these estimated associations were slightly larger in magnitude. In the SRS study, the estimated ERRs of malignant lymphoma per sievert under 5- and 10-year lag assumptions were 6.99 (90% CI: 0.96, 18.39) and 8.18 (90% CI: 1.44, 21.16), respectively. Upon exclusion of deaths due to

Table 1. Observed Numbers of Deaths Due to Malignant Lymphoma Among Male Atomic Bomb Survivors (1950–2000) and Male Workers at the Savannah River Site (1950–2002), by Age Group, Japan and South Carolina[a]

Attained Age, years	Atomic Bomb Survivors[b]			Savannah River Site Workers		
	Person-Years of Follow-Up	No. of Deaths		Person-Years of Follow-Up	No. of Deaths	
		Malignant Lymphoma	Non-Hodgkin's Lymphoma		Malignant Lymphoma	Non-Hodgkin's Lymphoma
<35	50,103	1	1	119,174	2	2
35–39	31,253	2	1	66,573	2	2
40–44	39,991	3	2	66,937	2	2
45–49	50,727	3	3	61,141	0	0
50–54	63,495	6	4	53,782	3	3
55–59	73,109	4	4	47,115	4	4
60–64	76,830	9	9	41,019	4	3
65–69	74,314	14	13	33,865	15	11
70–74	58,446	19	19	21,880	15	15
75–79	37,956	17	16	9,712	5	5
≥80	35,138	12	12	4,494	4	4
Total	591,359	90	84	525,691	56	51

[a] Because of rounding, column totals for person-time differ slightly from the sums of rows.
[b] Japanese males who were aged 15–64 years and present in Hiroshima or Nagasaki at the time of the bombings.

Hodgkin's disease, these estimated associations were slightly smaller in magnitude. The SRS cohort included a single death due to malignant lymphoma among black workers; upon restriction to nonblack workers, the estimated ERRs of malignant lymphoma per sievert under 5- and 10-year lag assumptions were 7.10 (90% CI: 1.00, 18.66) and 8.18 (90% CI: 1.44, 21.16), respectively.

When the LSS data were limited to survivors with doses in the range of 0–<0.5 Sv, estimates of radiation-lymphoma mortality associations were of greater magnitude than estimates obtained from model fittings over the entire dose range. Under a 5-year lag assumption, the estimated ERRs of malignant lymphoma and NHL per sievert were 3.02 (90% CI: 0.33, 7.22) and 2.86 (90% CI: 0.10, 7.24), respectively. While this suggests nonlinearity in the dose-response association, comparison of a linear-quadratic dose-response function with a purely linear dose-response function indicated that inclusion of a quadratic term resulted in very little improvement in model fit (LRT = 0.07, 1 df; $P = 0.79$). Under a 10-year lag assumption, the estimated ERRs of malignant lymphoma and NHL per sievert were 4.54 (90% CI: 1.16, 9.93) and 4.24 (90% CI: 0.83, 9.76), respectively.

In the LSS, there was no evidence of an association between radiation dose and lymphoma mortality during the periods 5–25 years or 26–35 years after irradiation (Table 3). Positive associations between lymphoma mortality and dose were observed during the periods 36–45 years and 46–55 years after irradiation. Analyses of associations between radiation dose and NHL led to risk estimates similar to those obtained via analyses of all malignant lymphoma (Table 3). In a nested model, defined post hoc, we evaluated the association between dose and malignant lymphoma mortality during the periods 5–35 years postexposure and 36–55 years postexposure. There was no evidence of association 5–35 years after exposure (ERR/Sv = 0.03, 90% CI: ND, 1.15; LRT = 0.00, $P = 0.96$); however, there was a positive

Table 2. Estimated Association Between Lymphoma Mortality and Ionizing Radiation Dose Under 5- and 10-Year Exposure Lags Among Male Atomic Bomb Survivors (1950–2000) and Male Workers at the Savannah River Site (1950–2002), Japan and South Carolina

Exposure Lag and ERR	Atomic Bomb Survivors[a]		Savannah River Site Workers	
	Malignant Lymphoma	Non-Hodgkin's Lymphoma	Malignant Lymphoma	Non-Hodgkin's Lymphoma
5 years				
ERR per Sv	0.79	0.86	6.99	6.45
90% CI	0.10, 1.88	0.13, 2.03	0.96, 18.39	0.48, 17.95
P value[b]	0.05	0.04	0.04	0.07
10 years				
ERR per Sv	1.06	1.12	8.18	7.62
90% CI	0.24, 2.38	0.26, 2.51	1.44, 21.16	0.93, 20.77
P value	0.02	0.02	0.03	0.05

Abbreviations: CI, confidence interval; ERR, excess relative rate.
[a] Japanese males who were aged 15–64 years and present in Hiroshima or Nagasaki at the time of the bombings.
[b] *P* value from a likelihood ratio test that the reported parameter for the estimated ERR was equal to 0.

Table 3. Estimated Association Between Radiation Dose and Lymphoma Mortality Among Male Atomic Bomb Survivors,[a] by Time Since Exposure, Hiroshima and Nagasaki, Japan, 1950–2000

Lymphoma Type and ERR	Time Since Exposure, years (Calendar Period)			
	5–25 (1950–1970)	26–35 (1971–1980)	36–45 (1981–1990)	46–55 (1991–2000)
Malignant lymphoma				
ERR per Sv	0.08	−0.10	2.23	1.70
90% CI	ND, ND	ND, ND	0.09, 6.91	0.16, 5.36
P value[b]	0.89	0.91	0.08	0.05
No. of deaths	31	20	16	23
Non-Hodgkin's lymphoma				
ERR per Sv	0.17	−0.10	2.23	1.70
90% CI	ND, ND	ND, ND	0.09, 6.91	0.16, 5.36
P value	0.79	0.91	0.08	0.05
No. of deaths	25	20	16	23

Abbreviations: CI, confidence interval; ERR, excess relative rate; ND, not determined.

[a] Japanese males who were aged 15–64 years and present in Hiroshima or Nagasaki at the time of the bombings.

[b] *P* value from a likelihood ratio test that the reported parameter for the estimated ERR was equal to 0.

association between dose and lymphoma mortality ≥36 years after exposure (ERR/Sv = 1.93, 90% CI: 0.48, 4.66; LRT = 6.83, $P < 0.01$).

In analyses of the SRS cohort, there was a highly imprecise positive association between lymphoma mortality and doses accrued during the periods 5–25 and 26–35 years prior. The association with doses accrued ≥36 years prior was of the largest magnitude and contributed most to the goodness of model fit. The estimated dose-response association within each exposure time window was based on the total number of lymphoma deaths. Similar estimates were obtained in analyses restricted to NHL (Table 4).

When the LSS data were limited to those survivors with doses in the range of 0–<0.5 Sv, there were positive, albeit imprecise, estimates of association between radiation dose and malignant lymphoma mortality during the periods 5–25 years after irradiation (ERR/Sv = 0.64, 90% CI: −1.69, 5.94; LRT = 0.1, $P = 0.75$), 26–35 years after irradiation (ERR/Sv = 2.52, 90% CI: −1.48, 11.71; LRT = 0.7, $P = 0.40$), 36–45 years (ERR/Sv = 7.08, 90% CI: −0.08, 22.86; LRT = 2.6, $P = 0.11$), and 46–55 years after irradiation (ERR/Sv = 6.42, 90% CI: −0.22, 23.11; LRT = 2.4, $P = 0.12$). Results for analyses of NHL were similar to those for all lymphoma mortality. There was a negative association between radiation dose and NHL mortality during the period 5–25 years after irradiation (ERR/Sv = −0.41, 90% CI: ND, 5.00; LRT = 0.03, $P = 0.85$) and positive associations between radiation dose and mortality during the periods 26–35 years after irradiation (ERR/Sv = 2.46, 90% CI: −1.50, 11.55; LRT = 0.68, $P = 0.41$), 36–45 years after irradiation (ERR/Sv = 7.07, 90% CI: −0.08, 22.83; LRT = 2.61, $P = 0.11$), and 46–55 years after irradiation (ERR/Sv = 6.42, 90% CI: −0.23, 23.11; LRT = 2.41, $P = 0.12$).

Table 5 shows observed and expected numbers of malignant lymphoma deaths by dose category under 5- and 10-year lag assumptions. The distribution of events among SRS workers with respect to dose was relatively narrow in comparison with the LSS data. Over the dose range at which the ratio of observed to expected numbers of malignant lymphoma deaths could be compared in these 2 cohorts (i.e., 0–<0.5 Sv), these ratios were similar in magnitude for analyses of the 2 cohorts, although values tended to be slightly greater for the SRS cohort than for the LSS cohort. Ratios of observed to expected numbers of deaths were

Table 4. Estimated Association Between Radiation Dose and Lymphoma Mortality Among Male Workers at the Savannah River Site, by Time Since Exposure, South Carolina, 1950–2002

Lymphoma Type and ERR	Time Since Exposure, years		
	5–25	26–35	36–52
Malignant lymphoma			
ERR per Sv	1.18	4.06	33.28
90% CI	ND, ND	ND, 25.34	4.83, 107.9
P value[a]	0.85	0.64	0.03
Non-Hodgkin's lymphoma			
ERR per Sv	1.51	0.58	38.35
90% CI	ND, 16.02	ND, 22.83	7.02, 121.57
P value	0.80	0.95	0.02

Abbreviations: CI, confidence interval; ERR, excess relative rate; ND, not determined.

[a] *P* value from a likelihood ratio test that the reported parameter for the estimated ERR was equal to 0.

Table 5. Observed and Expected Numbers of Deaths Due to Malignant Lymphoma Among Male Atomic Bomb Survivors (1950–2000) and Male Workers at the Savannah River Site (1950–2002), by Radiation Dose, Japan and South Carolina[a]

Assumed Lag and Cohort	Radiation Dose, Sv						
	<0.005	0.005–<0.10	0.10–<0.20	0.20–<0.50	0.50–<1	1–<2	≥2
5-year lag							
Atomic bomb survivors[b]							
No. of deaths observed	32	29	8	11	3	5	2
Obs/Exp ratio[c]	0.80	0.97	1.33	1.61	0.72	2.04	2.60
Mean dose, Sv	0.001	0.032	0.141	0.322	0.721	1.340	2.392
Person-years of follow-up	260,641	195,354	38,255	45,932	28,566	16,674	5,937
Savannah River Site workers							
No. of deaths observed	20	24	7	5	0	0	0
Obs/Exp ratio	0.77	1.01	1.78	2.14			
Mean dose, Sv	0.001	0.028	0.142	0.266			
Person-years of follow-up	305,131	181,767	25,961	12,830	0	0	0
10-year lag							
Atomic bomb survivors							
No. of deaths observed	27	27	8	11	3	5	2
Obs/Exp ratio	0.73	0.97	1.44	1.73	0.78	2.19	2.73
Mean dose, Sv	0.001	0.032	0.141	0.322	0.722	1.338	2.392
Person-years of follow-up	213,808	160,274	31,330	37,840	23,545	13,827	4,926
Savannah River Site workers							
No. of deaths observed	21	24	6	5	0	0	0
Obs/Exp ratio	0.77	1.05	1.60	2.35			
Mean dose, Sv	0.001	0.028	0.141	0.264			
Person-years of follow-up	344,948	149,706	21,197	9,840	0	0	0

Abbreviations: Exp, expected; Obs, observed.
[a] Because of rounding, some column totals for person-time differ slightly from the sums of rows.
[b] Japanese males who were aged 15–64 years and present in Hiroshima or Nagasaki at the time of the bombings.
[c] Ratio of the number of deaths observed to the number of deaths expected.

minimally affected by exclusion of deaths due to Hodgkin's disease (results not shown).

DISCUSSION

In a previous analysis of lymphoma mortality among survivors in the LSS, Pierce et al. (17) reported evidence of a nonsignificant positive association with radiation dose among males (ERR/Sv = 0.27, 90% CI: ND, 1.49) and a nonsignificant negative association among females (ERR/Sv = −0.17, 90% CI: ND, 0.30). In those analyses, a time-constant ERR model was fitted to mortality follow-up through 1990. In the present paper, time-window analyses helped to explain the observation of a significant positive association between radiation dose and lymphoma mortality among male atomic bomb survivors with more recent follow-up, showing that positive associations have been observed only since 1980. Such findings suggest a protracted induction and latency period. If considered within the framework of a multistage model of carcinogenesis, the relatively long empirical induction period for lymphoma following radiation exposure may be consistent with action at an early stage of a multistage process.

The point estimates for the radiation dose-lymphoma mortality association under 5- and 10-year lag assumptions derived from analysis of the SRS cohort are larger than the estimates derived from analysis of the LSS cohort (Table 2). Differences in the magnitude and rate of exposure may influence the comparability of dose-response estimates. These cohorts also differ with regard to potential biases from confounding, selection, and exposure measurement error. While it is not an established cause of NHL, benzene is suspected to be related to NHL (28). However, benzene was not used in the production process at SRS, nor was it routinely used as a degreaser. Plutonium-239 is a radiologic hazard at SRS. While a recent study suggested that the contribution of plutonium doses to total dose estimates for these workers was relatively small (29), we did not directly assess confounding by plutonium exposure. Selection bias could have influenced these estimates of association—for example, via the "healthy worker" survivor effect (20). Although we adjusted for employment status, such an approach is suboptimal if employment status is an intermediate variable

as well as a confounder of the association of interest. However, in studies of chronic diseases with long latency periods, cumulative exposure will typically not appreciably influence employment termination rates; under such conditions, employment status will play a minor role as an intermediate variable but could have a strong role as a confounder of the association (22). Frequent reading of dosimeters could have led to dose underestimation if dosimeters were not sufficiently exposed to reach a minimum detectable dose. However, prior work suggests that the impact of this source of measurement error on estimates of radiation dose-response trends is modest (30–32).

Problems of bias could also influence estimates of radiation-mortality associations among atomic bomb survivors. DS02 estimates account for the initial radiation released from the detonation of the weapons but not radiation from fallout or neutron activation of the ground and structures (33). The available data suggest that most people in Hiroshima and Nagasaki had low cumulative external doses from fallout, with maximum estimates in the range of 0.2–0.4 Sv for several hundred people who were in an area of Nagasaki approximately 3 km from the hypocenter (33, 34). Selective survival in the LSS cohort is another concern and is a generic consideration when trying to understand the temporal evolution of exposure-related risk (35). A relation between short-term survival after the bombings and later risk of lymphoma could lead to bias in dose-response estimates. Evidence of selection has been suggested by some empirical analyses (36, 37); however, values for the magnitude of dose-related selective survival assumed in a recent study suggested a modest potential for bias in dose-response estimates (38).

These analyses provide evidence of a positive association between ionizing radiation dose and malignant lymphoma mortality among male Japanese atomic bomb survivors and SRS workers. We did not address risk estimates for females, for whom there was no evidence of a positive association between radiation dose and lymphoma mortality in follow-up through 1990 (17). The radiation-NHL mortality associations among these male atomic bomb survivors and SRS workers are of larger magnitude than the estimate reported in a 15-country study of nuclear workers (under a 10-year lag assumption, ERR/Sv = 0.44, 90% CI: <0, 4.78) (7); however, in the current analyses, positive dose-response associations were primarily observed more than 35 years after irradiation. These findings underscore the importance of continued follow-up of the LSS cohort and nuclear worker cohorts.

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Ionizing Radiation and Leukemia Mortality among Japanese Atomic Bomb Survivors, 1950–2000

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This paper provides the first comprehensive report on mortality by type of leukemia among the Japanese atomic bomb survivors in the Life Span Study (LSS). Analyses include 310 deaths due to leukemia during the period 1950–2000 among 86,611 people in the LSS. Poisson regression methods were used to evaluate associations between estimated bone marrow dose and leukemia mortality. Attention was given to variation in the radiation dose–leukemia mortality association by time since exposure, age at exposure, city and sex. The excess relative rate per gray of acute myeloid leukemia was best described by a quadratic dose–response function that peaked approximately 10 years after exposure. Acute lymphatic leukemia and chronic myeloid leukemia mortality were best described by a linear dose–response function that did not vary with time since exposure. Adult T-cell leukemia was not associated with estimated bone marrow dose. Overall, 103 of the 310 observed leukemia deaths were estimated to be excess deaths due to radiation exposure. In the most recent decade of observation (1991–2000), the estimated attributable fraction of leukemia deaths among those survivors exposed to >0.005 Gy was 0.34, suggesting that the effect of the atomic bombings on leukemia mortality has persisted in this cohort for more than five decades.

INTRODUCTION

Atomic bombs were detonated over the cities of Hiroshima and Nagasaki on August 6, 1945 and August 9, 1945, respectively. In each city, tens of thousands died on the day of the bombing. In the weeks immediately after the bombings, many survivors fell ill and died, succumbing to burns, bone marrow depletion, and other consequences of the blast, thermal radiation and ionizing radiation (*1, 2*). The U.S. Atomic Bomb Casualty Commission (ABCC) had a particular interest in the hematological consequences of the atomic bombings and established a study of hematological conditions among the atomic bomb survivors shortly after the creation of the ABCC in 1947. By the late 1940s there was evidence of an excess of leukemia among the Japanese atomic bomb survivors (*3*). A 1955 review of the ABCC's work led to the initiation of a large population-based study of mortality and disease risk in relation to the survivors' distance from the hypocenters of the atomic bombings (*4, 5*). That study, known as the Life Span Study (LSS), became the foundation for much of the ongoing research on mortality and cancer incidence among the Japanese atomic bomb survivors (*6, 7*).

To date, published analyses of mortality among the LSS members have considered the risk of death due to leukemia of all types in aggregate (*8–10*). In contrast, analyses of cancer incidence in the LSS cohort have examined the risk of leukemia in aggregate and by subtype (*6, 11, 12*). Although cancer incidence studies offer advantages relative to analyses that use death certificate information, cause-of-death information is collected for all decedents in the LSS while information on cancer incidence is ascertained systematically only for those survivors residing in the catchment areas for the Hiroshima and Nagasaki cancer registries (Hiroshima prefecture and Nagasaki prefecture, respectively). Cancer incidence analyses can only indirectly account for the effect of migration out of the catchment areas on the completeness of case ascertainment by means of city-, sex-, age- and period-specific estimates of migration probabilities (*6*). Furthermore, the Hiroshima and Nagasaki tumor registries were not established until 1957 and 1958, respectively. Although the ABCC established a special leukemia registry for atomic bomb survivors in 1947, the protocols followed by that leukemia registry differed from the protocols employed

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by the contemporary tumor registries. In the early years of the leukemia registry, cases of leukemia and related disorders were identified from a variety of sources, including death certificates and newspaper reports for Hiroshima, Nagasaki and the surrounding areas, ABCC clinical records, and autopsy records; cases were coded according to an *ad hoc* classification system after review by an ABCC hematologist (*6, 13*). Given the systematic collection of cause of death data for LSS members since the cohort's enumeration in 1950, the inherent uncertainty in estimates of survivors' migration histories, and the interest in characterizing the leukemia risks for all survivors in the LSS regardless of their subsequent place of residence, the information contributed by a leukemia mortality analysis is important and fills a gap that the cancer incidence data cannot address. This paper reports on the risk of radiation-related mortality by type of leukemia among the Japanese atomic bomb survivors in the LSS.

MATERIALS AND METHODS

The LSS of atomic bomb survivors includes 86,611 people who were present in Hiroshima or Nagasaki at the time of the bombings, were residents of the city at the time of the 1950 census, and have dose estimates based upon the DS02 dosimetry system (*9*). LSS members who were away from the cities at the time of the bombings were excluded from this analysis.

Vital status and cause of death information have been collected continually since the cohort's inception; these analyses examine follow-up data spanning the period October 1, 1950 through December 31, 2000. Classification of decedents was according to underlying cause of death coded to the 7th revision of the International Classification of Diseases (ICD7) for deaths coded in 1950–1967, the 8th revision (ICD8) for deaths coded in 1968–1978, the 9th revision (ICD9) for deaths coded in 1979–1997, and the 10th revision of (ICD10) for deaths coded since 1998. Cause-of-death information originally coded to ICD7 was recoded to ICD9 to permit classification of decedents according to more contemporary categories of cause of death. This analysis considers the following categories of cause of death: leukemia of all types (ICD8 codes 204–205; ICD9 codes 204–208; ICD10 codes C91–C95); acute lymphatic leukemia, ALL (ICD8 code 204.0; ICD9 codes 204.0, 204.2; ICD10 codes C91.0, C91.2); acute myeloid leukemia, AML (ICD8 code 205.0; ICD9 codes 205.0, 205.2; ICD10 codes C92.0, C92.2, C92.4, C92.5); chronic myeloid leukemia, CML (ICD8 code 205.1; ICD9 code 205.1; ICD10 code C92.1); and adult T-cell leukemia, ATL (ICD10 code C91.5). ATL began to be noted as a disease entity on the death certificate in the 1980s; however, ATL was not assigned a unique ICD code prior to the 10th revision. For deaths coded to earlier revisions of the ICD, ATL cases were identified by manual review of death certificates. Since only seven deaths were attributed to chronic lymphocytic leukemia (CLL), we did not examine separate dose-response associations for this type of leukemia.

The primary exposure of interest was defined as weighted DS02 bone marrow dose adjusted for dosimetry errors (*14*). Individual dose estimates for survivors within 2 km of the bombings were based on estimates of penetrating radiation emitted by the bombs and locations and shielding of survivors derived from interviews conducted in the late 1950s and early 1960s. Dose estimates for other survivors are based on less detailed information on shielding provided through interviews. Uncertainties about survivor location and shielding are an important potential source of error in individual dose estimates. Adjusted dose estimates have been developed to compensate for attenuation bias due to random errors in these dose estimates, with shielded kerma estimates above 4 Gy truncated to 4 Gy (*15*). Doses are expressed as the weighted dose in grays and represent the sum of the γ-radiation dose plus the neutron dose multiplied by 10, since it is assumed that neutron doses have a greater effectiveness than γ rays at increasing the incidence of leukemia.

Statistical Methods

The analytical data file for these analyses consists of a table of person-time and leukemia deaths cross-classified by city (Hiroshima or Nagasaki), sex, attained age (in 5-year intervals), age at exposure (which is equivalent to birth cohort, in 5-year intervals), calendar time (1958–1960, then in 5-year intervals up to 1985, the final categories being 1986–1987, 1988–1990, 1991–1995, and 1996–1998), location at the time of the bombing (within 3 km or 3–10 km from the hypocenter), and bone marrow dose (<0.005, 0.005–<0.02, 0.02–<0.04, 0.04–<0.06, 0.06–<0.08, 0.08–<0.1, 0.1–<0.125, 0.125–<0.150, 0.150–<0.175, 0.175–<0.2, 0.2–<0.25, 0.25–<0.3, 0.3–<0.5, 0.5–<0.75, 0.75–<1, 1–<1.25, 1.25–<1.5, 1.5–<1.75, 1.75–<2, 2–<2.5, 2.5–<3, 3+ Gy). For each cell of the cross-classification, the number of observed leukemia deaths (total and by subtype), the number of person-years, and person-year weighted average values for dose, attained age and age at exposure were computed.

Radiation dose–mortality associations were estimated by a regression model of the form rate $= e^{\alpha_i}[\,[1 + ERR(d,c,s,e,t)]$, where α_i indexes strata defined by city, sex, attained age, birth cohort (<1895, 1895–1904, 1905–1914, 1915–1924, 1925–1945), and location at the time of the bombing, d represents the estimated radiation dose delivered at the time of bombings in August 1945, and c, s, e and t denote city, sex, age at exposure and time since exposure, respectively.

The excess relative rate of leukemia was described by a model of the form $ERR(d,c,s,e,t) = \rho(d)\,\varepsilon(c,s,e,t)$, where $\rho(d)$ describes the shape of radiation dose–response function and $\varepsilon(c,s,e,t)$ describes modifiers of the radiation dose effect. A model with a linear radiation dose–response function, $\rho(d) = \beta d$, was compared to a model with a linear-quadratic dose–response function, $\rho(d) = (\beta d + \theta d^2)$, or a purely quadratic dose–response function, $\rho(d) = \theta d^2$.

The modifying effect of age at exposure and time since exposure was described with a multiplicative model such as $\varepsilon(e,t) = \exp[\gamma f(e) + \delta g(t) + \phi f(e)g(t)]$. Effect modification by age at exposure was modeled as $f(e) = \min(0, (e - 30)/10)$, denoted as e' for convenience. The effect of time since exposure was parameterized as $g(t) = t$ or $g(t) = \log(t)$. In addition to these approaches, we evaluated models that allowed non-monotonic functions of time since exposure by inclusion of indicator variables for categories of time since exposure and via a cubic spline function of time since exposure with join points (i.e. knots) at 15, 30 and 45 years after exposure (*16, 17*). The knot locations were chosen to partition the follow-up period, which commenced 5 years after exposure, into intervals of 15 years or less. Cubic splines are flexible, piecewise polynomials that can be estimated with standard regression programs. Unlike lower-order splines, cubic splines can describe a wide variety of functional forms with a small number of knots (*16*). Splines with fewer knots tend to imply smoother functions; where appropriate we fitted reduced models with fewer knots, with judgment regarding the optimal number of knots based on evaluation of the residual model deviance. Evaluation of effect measure modification by sex or city was achieved by including a linear product term for the factor with a model such as $\varepsilon(c,e,t) = \omega_c \exp[\gamma f(e) + \delta g(t) + \phi f(e)g(t)]$.

Parameter estimation was carried out using the AMFIT program in the EPICURE statistical package (*18*). For consistency with other epidemiological studies of radiation-exposed populations, 90% confidence intervals were generated for estimated parameters via the profile likelihood method (*19*). To aid interpretation of some model fittings, likelihood ratio test (LRT) statistics and associated

one-sided P values are reported. Akaike's Information Criterion (AIC) is used to inform model selection when comparing two or more non-nested models, with AIC = $-2LogL + 2k$, where k is the number of parameters in the statistical model and $-2LogL$ is the deviance for the fitted model. For a set of competing models, the preferred model minimizes the AIC. In addition, the estimated numbers of background and excess cases are provided for some model fittings. These estimates are the sums of cell-specific values computed from the final risk model for the outcome of interest; the background cases are obtained by multiplying cell-specific person-time counts by the stratum-specific baseline rate estimates for the fitted model and the excess cases defined as the difference between the estimated number of background cases and the total number of cases expected under the fitted model. The ratio of the estimated number of excess cases to the total number of fitted cases among survivors with estimated doses exceeding 0.005 Gy is reported as the attributable fraction of deaths among survivors with doses exceeding 0.005 Gy and is denoted $AF_{0.005\,Gy}$ (*20*). The time-averaged estimate of the excess absolute rate of leukemia, denoted EAR, is calculated as the ratio of the number of excess deaths to the total number of person-year Gy in the cohort to date.

RESULTS

Table 1 describes the distribution of person-time and leukemia deaths by city, sex and categories of attained age, age at the time of bombing, and calendar period. Among cohort members from Hiroshima, the most common type of leukemia was AML while the least common type of leukemia was ATL. Among cohort members from Nagasaki, the least common subtype of leukemia was ALL. No deaths due to ATL were observed among people less than 50 years of age, and no cases of ATL were noted prior to 1981.

There were 94 deaths due to leukemia that were not classified as AML, CML, ALL or ATL. These include seven deaths due to CLL, one death due to other/unspecified forms of lymphatic leukemia (ICD9 codes 204. 8, 204.9), 12 deaths due to other/unspecified forms of myeloid leukemia (ICD9 codes 205.3, 205.8, 205.9), three deaths classified as chronic leukemia not otherwise specified (ICD9 code 208.1), 25 deaths classified as acute leukemia not otherwise specified (ICD 9 codes 208.0, 208.2), and 46 deaths classified as other/unspecified leukemia (ICD9 codes 206, 207, 208.8 and 208.9).

Leukemia: All Types

Table 2 presents the distribution of person-time and leukemia deaths by category of estimated marrow dose, as well as indicating the person-time weighted average distance from hypocenter for each estimated dose category. The largest numbers of person-years at risk and leukemia deaths were observed among survivors in the lowest estimated dose category (<0.005 Gy) who were, on average, 4007 m from the hypocenters.

Leukemia mortality rate ratios were estimated by categories of bone marrow dose, with people who had doses <0.005 Gy serving as the reference category. Rate ratios were greater than unity among people in the highest four estimated dose categories. Also shown in Table 2 is the estimated dose–response association derived with a linear ERR model without effect modification. There is a positive association between estimated dose and leukemia mortality (LRT = 170.3, 1 *df*, $P < 0.001$). The fit of the regression model improved upon inclusion of a quadratic term to the dose–response function (LRT = 7.9, 1 *df*, $P = 0.005$). Analyses of effect modification, described below, were based upon a model with a linear-quadratic radiation dose–response function.

Exploratory analyses showed that the effect of age at exposure was modeled parsimoniously with the continuous term $e' = \min[0, (e - 30)/10]$. Examination of the data suggested that the effect of time since exposure diverged from a simple monotonic function (Appendix Fig. A2). Time since exposure was therefore described by a cubic spline function with knots at 15, 30 and 45 years after exposure. It was found necessary to allow the

TABLE 1
Distribution of Person-Time and Deaths due to Leukemia by City, Sex, Attained Age, Age at Time of Bombing, and Calendar Period

	Leukemia					
	All types	AML	CML	ALL	ATL	Person-years/ 10^4
City						
Hiroshima	227	94	50	17	1	212.6
Nagasaki	83	30	8	2	14	105.8
Sex						
Male	165	61	33	8	9	124.0
Female	145	63	25	11	6	194.4
Attained age (years)						
5–9	4	0	1	1	0	2.3
10–19	13	4	2	2	0	17.6
20–29	22	6	3	0	0	34.1
30–39	22	8	7	0	0	44.8
40–49	37	16	10	3	0	55.6
50–59	44	16	5	2	5	63.7
60–69	75	29	19	7	5	52.8
70+	93	45	11	4	5	47.5
Age at exposure (years)						
0–9	43	14	10	3	2	86.4
10–19	67	27	10	8	7	81.1
20–29	50	24	4	2	3	47.6
30–39	60	25	14	4	1	45.6
40–49	59	24	12	1	1	36.5
50+	31	10	8	1	1	21.3
Calendar period						
Oct. 1950–1960	81	26	17	3	0	84.4
1961–1970	48	15	13	2	0	73.1
1971–1980	62	30	12	4	0	63.6
1981–1990	62	26	12	5	5	53.7
1991–2000	57	27	4	5	10	43.7
Distance (km)						
0–3 km	239	101	46	14	11	225.1
3–10 km	71	23	12	5	4	93.3