lated as the sum of the gamma ray dose plus 10 times the neutron dose. The factor 10 represented the relative biological effectiveness (RBE) of neutrons, because neutrons were suspected to have a greater biological effect than gamma rays [18]. Adjusted doses were used to reduce bias in risk estimates.

2.3. Dietary assessment

A lifestyle questionnaire was sent to the LSS participants between June and November 1979, and the mail survey was completed on January 1, 1980 for men, and February 1, 1981 for women [19]. The questionnaire included items on past medical history, marital status, anthropometrical information, smoking and drinking habits, a 22-item food frequency questionnaire (FFQ), occupation, and education.

In the present study, we used the answers to two dietary factors: green–yellow vegetables and fruit. The average consumption within the previous year was categorized into "never and once a week or less", "twice to four times a week", and "daily or almost daily". The validity of the FFQ has been previously reported, showing moderate correlation with the 24-h diary used as reference method [21].

2.4. Follow-up of mortality

The follow-up carried out regular checks on the vital status of the LSS participants, through the Japanese family registration system (Koseki). The Koseki provides complete coverage for all LSS members residing in Japan [18]. Copies of death certificates are regularly obtained by RERF for all deceased LSS participants, and trained coders enter the appropriate codes into the database. Causes of death were coded according to the International Classification of Diseases, ninth and tenth revisions [22]. The follow-up was started on January 1, 1980 for men and February 1, 1981 for women, and continued until the date of death or December 31, 1999, whichever came first.

We previously reported that consumption of green–yellow vegetables and fruit had a significant protective effect on total cancer, stomach, liver and lung cancer mortality [3]. Therefore, the current analyses will focus on total solid cancer and the three significant sites.

2.5. Statistical analysis

To examine the joint effects of vegetables or fruit consumption and radiation exposure on cancer risk, the following two hazard risk models were applied using the dummy variables, m for moderate, and h for high consumption of vegetables or fruit:

- Additive model:

$$\text{Relative risk} = e^{\gamma Z}(1 + \beta_1 m + \beta_2 h + \beta_3 d)$$

- Multiplicative model:

$$\text{Relative risk} = e^{\gamma Z}(1 + \beta_1 m + \beta_2 h)(1 + \beta_3 d)$$

where Z represents other cancer risk factors: city (Hiroshima, Nagasaki); sex; age at the start of follow-up; smoking habits (never, current, past); drinking habits (never, current, past); and education level (low, middle, high); and where $m = 1$ for moderate intake group of vegetables or fruit, and 0 for low and high intake groups; $h = 1$ for high intake group of vegetables or fruit, and 0 for low and moderate intake groups; d is the radiation dose in Sievert.

For each model, the test for data fitting was measured in comparing the deviances between the additive and the multiplicative models.

Relative risks were estimated using the PEANUTS procedure of the EPICURE software [23].

3. Results

The total study population included 13,309 men, and 21,509 women. The mean age was 55 years (range = 34–98 years).

During the follow-up period of 20 years, 2628 cancer deaths were identified, including 522 deaths due to stomach cancer, 477 liver cancer deaths, and 458 lung cancer deaths.

Table 2 shows the percentage consumption of green–yellow vegetables and fruit, according to the radiation dose exposure. Most of the participants had vegetables twice to four times a week, and fruit everyday. The distribution was similar across the radiation exposure categories.

Table 3 presents the percentage change in cancer risk, according to vegetables and fruit intake, and to

Table 2
Intake distribution (in percentage) by radiation dose

Dose (Sv)	Green–yellow vegetables			Fruit		
	≤1/week	2–4/week	Daily	≤1/week	2–4/week	Daily
0 (≤0.005)	31.7	43.8	24.5	23.5	31.0	45.5
0.005–0.5	32.0	43.1	24.9	24.3	31.5	44.2
0.5–1.0	32.6	44.3	23.1	25.0	31.7	43.3
>1.0	35.0	43.5	21.5	25.2	32.9	41.9

radiation exposure, in two different interaction models. The first model, the additive model, implies that the combined effects of diet and radiation on the cancer risk is the sum of the effect of diet in non-exposed persons, and the effects of radiation in subjects with low intake of the dietary factor. The second model, the multiplicative model, refers to the combined effects of diet and radiation that is the product of their effects alone; in other words, the higher dose a person received, the more effective the diet.

For solid cancer, in the additive model, the persons with a daily consumption of vegetables or fruit, without any exposure to radiation, had a 13% decreased risk of cancer. Those exposed at 1 Sv, and with a low consumption of vegetables or fruit, had a 48–49% increased risk of cancer. The joint-effects showed consequently a lower risk of cancer among those exposed to 1 Sv who had a diet high in vegetables and fruit, as compared to those with a low diet (vegetables: risk of 36% instead of 49%). The multiplicative model gave similar results. The effect of a diet rich in vegetables or fruits decreased the risk of cancer due to radiation from 52 to 32–34%.

As for specific cancer sites, the effect of a daily intake of vegetables or fruit was through a reduction of cancer risk. The results are consistent with previous findings in the same cohort, where we showed that fruit decreased significantly the risk of stomach cancer, and vegetables the risk of liver cancer [3]. Not surprisingly, the risk of cancers increased significantly with radiation exposure; and the joint-effects of diet and radiation showed a lower risk of cancer as compared to the effects of radiation only, suggesting that a diet rich in vegetables and fruit benefits persons exposed to atomic radiation. We also checked the multiplicative joint-effects for the specific cancer sites. The effects were similar to the additive effects, consequently the results are not shown.

For the additive and multiplicative models, the test for lack of fit showed a P-value greater than 0.5. This means that there is no significant evidence for rejecting either the additive or the multiplicative model.

4. Discussion

A diet rich in green–yellow vegetables and fruit reduced the risk of cancer among those exposed to ionising radiation. However, we were not able to rule out that there was any additive or multiplicative effect of diet on the radiation effect. The main reason was that the data did not have enough power. Most of the study subjects included in the LSS cohort received a very low radiation dose. Those exposed to 1 Sv or more represented less than 3% of the population (see Table 1). The radiation effect in this cohort has a low power. It explains only 5% of the cancer risks because most of the survivors received a low radiation dose [1]. Additionally, the effects of diet on cancer risk in human beings are generally low. Thus, in the present population, it was difficult to show any risk difference modification at a significant level. Nevertheless, despite the low power of the data, the findings cannot exclude a post-irradiation, long-term protective effect of dietary factors on cancer risk.

Studies on effects of diet on cancer incidence or mortality in radiation-exposed persons are limited [11]. One study reported that a high intake of fresh fruit and vegetables was associated with an increased immune protection in young children exposed to the Chernobyl accident. The protective effects were observed 3–7 years after the radiation exposure [24].

Fruit and vegetables are a rich source of antioxidants. Several clinical trials on the effects of antioxidant nutrients on chronic radiation damage among therapeutically irradiated persons have been pub-

Table 3
Percentage of change in risk of cancer, in additive and multiplicative models, according to the dietary factor

	Additive effects of diet and radiation dose					
	Green–yellow vegetables			Fruit		
	% Change	95% CI	*P*-value	% Change	95% CI	*P*-value
All solid cancers						
Effect of a high intake of dietary factor in non-exposed to radiation subjects	−13	(−22 −03)	0.0073	−13	(−22 −04)	0.0047
Effect of radiation exposure at 1 Sv with low intake of dietary factor	+49	(+32 +66)	0.0001	+48	(+31 +65)	0.0001
Effect of a high intake of dietary factor in radiation exposed subjects	+36			+35		
Stomach cancer						
Effect of a high intake of dietary factor in non-exposed to radiation subjects	−13	(−34 +08)	0.2190	−19	(−38 −01)	0.0394
Effect of radiation exposure at 1 Sv with low intake of dietary factor	+44	(+06 +83)	0.0229	+42	(+05 +79)	0.0240
Effect of a high intake of dietary factor in radiation exposed subjects	+31			+23		
Lung cancer						
Effect of a high intake of dietary factor in non-exposed to radiation subjects	−08	(−33 +17)	>0.5	−17	(−37 +04)	0.1070
Effect of radiation exposure at 1 Sv with low intake of dietary factor	+66	(+20 +112)	0.0051	+61	(+18 +104)	0.0051
Effect of a high intake of dietary factor in radiation exposed subjects	+58			+44		
Liver cancer						
Effect of a high intake of dietary factor in non-exposed to radiation subjects	−28	(−47 −09)	0.0039	−02	(−25 +21)	>0.5
Effect of radiation exposure at 1 Sv with low intake of dietary factor	+47	(+10 +84)	0.0121	+54	(+12 +96)	0.0108
Effect of a high intake of dietary factor in radiation exposed subjects	+19			+52		
	Multiplicative effect of diet and radiation dose					
All solid cancers						
Effect of a high intake of dietary factor in non-exposed to radiation subjects	−13	(−22 −04)	0.0056	−12	(−21 −04)	0.0051
Effect of radiation exposure at 1 Sv with low intake of dietary factor	+52	(+32 +72)	0.0001	+52	(+34 +70)	0.0001
Effect of a high intake of dietary factor in radiation exposed subjects	+32			+34		

lished. Oral supplementation of a combination of pentoxifylline (800 mg per day) and Vitamin E (1000 IU per day), for at least 6 months, reversed cutaneous radiation syndrome in patients who had radiotherapy for head, neck or breast cancers 0.5–30 years before [25,26]. The same combination of pentoxifylline and Vitamin E reduced fibroatrophic uterine lesions in women who were irradiated in the pelvic area, 25 years ago [27]. Similarly, supplementation of Vitamin E (1200 IU per day) and Vitamin C (1.5 g per day) improved the symptomatology of chronic radiation proctitis following radiotherapy for prostatic and gynaecological cancers [28].

At the biological level, two clinical trials on the effects of a supplementation of nutrients have been reported in persons exposed to radiation from the Cher-

nobyl accident. Ben-Amotz et al. [29] showed that beta-carotene supplementation reduced the blood level of oxidation. Emerit et al. [30] also demonstrated that supplementation of antioxidants from Ginkgo biloba leaves (containing flavonoids and terpenoids) for 2 months reduced the clastogenic activity of the plasma in Chernobyl liquidators. Studies on the existence of persistent clastogenic factors or genomic instability among irradiated persons have reported conflicting results. Some demonstrated the persistence of clastogenic factors [31,32], others showed no enhanced genomic instability [33–35].

Mechanisms of cancer protection by dietary nutrients that can modulate radiation responses are far from clear. Antioxidants and other dietary substances may interfere with DNA damage. However, it is unclear whether their anti-mutagenic properties have any anti-carcinogenic effect [11].

Although our study was performed in a general setting, the findings go into the same direction as the results of clinical trials, even if they are not as strong as trials. The main reason has been discussed earlier; several other arguments are discussed now.

First, the daily dietary antioxidant intakes of the usual diet were at a much lower dose than supplementation intakes of clinical trials. It has been estimated in Japan that, in 1980, the daily per capita intake of Vitamin E was 12 IU (1000–1200 IU in trials), and Vitamin C was 123 mg (1500 mg in trials) [36,37]. It is possible that the usual diet amount was too low to show any strong joint-effect with past radiation exposure. It should also be noted that information on personal vitamin supplementation was not available in the present cohort. Moreover, while our findings suggested a protective role for fruit and vegetables, we cannot assume whether antioxidants and/or micronutrients were the protective factors.

Second, diet was assessed 35 years after ionising radiation exposure. Previous clinical studies reported an effect after a shorter time lag. The cancer promotion might have already occurred, while it has been hypothesised that antioxidant vitamins were more effective at the early stage of the carcinogenesis, like the initiation or promotion stages [2,38,39]. It would have been more appropriate to measure the fruit and vegetables intake just after the radiation exposure, i.e. in August 1945. According to the National Nutrition Survey, during the war and post-war years, fruit and vegetables intake per capita per day in Japan was very low (fruit, 16 g per day; vegetables, 76 g per day in 1949) [40]. This suggests that during the cancer initiation—several decades before the onset—fruit and vegetables compounds were at a low level and might have been ineffective.

Finally, we cannot rule out dietary measurement errors. Diet information is based on the response to a single food frequency questionnaire. During the follow-up period of 16 years, fruit and vegetables consumption among the cohort participants has likely changed. In the general population, within the last two decades, fruit consumption has decreased (1980, 155 g per day; 1998, 116 g per day), while vegetables intake has increased (1980, 251 g per day; 1998, 261 g per day) [36]. Moreover, measurement errors might have been due to the absence of information on the fruit and green–yellow vegetables types, or on the portion size, preventing us from estimating the nutrient intake and consequently performing more accurate analyses.

In conclusion, in this large cohort of atomic-bomb survivors, a daily intake of fruit and vegetables benefited those exposed to radiation in reducing their risk of cancer, at least to the same extent as those not exposed to radiation.

Acknowledgements

We thank Dr. Dale Preston and Dr. Nori Nakamura for their valuable comments on the draft of the manuscript. The Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, Japan is a private, non-profit foundation funded by the Japanese Ministry of Health, Labour and Welfare (MHLW) and the U.S. Department of Energy (DOE), the latter through the National Academy of Sciences. This publication was supported by RERF Research Protocol RP # 14-78.

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地域がん登録事業と今後の課題

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●はじめに

平成15年の人口動態統計によると，がん（悪性新生物）は死因の第1位であり，全死因の30.5%を占めている．がん罹患数については人口動態統計ではなく，現在，34道府県市で実施されている地域がん登録によって把握されている．そのうち精度の高い地域のデータをもとに，「地域がん登録精度向上と活用に関する研究」班（主任研究者：津熊秀明）によって全国罹患率が推計され，毎年報告されている．

本稿では，地域がん登録事業について，その整備の取り組み，個人情報保護とのかかわりについて紹介し，最後にその課題について述べる．

❶地域がん登録事業の整備の取り組み

平成16年度から開始された第3次対がん総合戦略研究事業「がん予防対策のためのがん罹患・死亡動向の実態把握の研究」班（主任研究者：祖父江友孝）では，以下に示す8つの「目標と基準」を設定して地域がん登録を整備することを目指している（http://ncrp.ncc.go.jp参照）．すなわち，地域がん登録が今後10年で達成すべき条件を「目標」，ある時点で一定水準を満たしていると判断する条件を「基準」と称し，「基準」を定期的に確認して底上げすることによって，「目標」の達成をより確実にすることとしている．

(1) がん登録事業実施に関する公的承認を得ていること

(2) がん登録に必要な項目に関して，収集・管理・提供が可能なこと

(3) 登録の完全性に関する条件を満たしていること（登録漏れなどの量的な精度）

(4) 登録の即時性に関する条件を満たしていること（診断から集計までの迅速さ）

(5) 登録の品質に関する条件を満たしていること（登録項目における不詳割合の低さなどの質的な精度）

(6) 予後追跡調査を行い，追跡率が条件を満たしていること

(7) 報告書作成を行っていること

(8) 登録資料の研究的利用の手続きが整備されていること（資料の研究的利用）

冒頭で述べた「精度の高い」地域がん登録事業とは，「目標と基準(3)」にある登録の完全性をもとに選ばれている．完全性の指標としてDCO

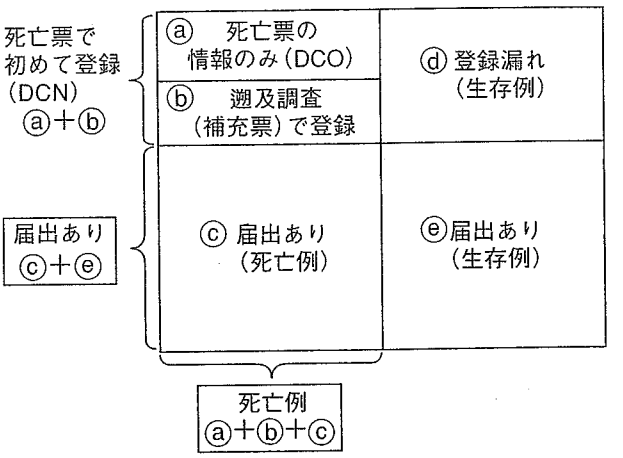

図1　地域がん登録事業の精度に関する模式図
地域がん登録では，まず届出（地域により出張採録方式も用いられる）によってがん患者を把握し（ⓒ+ⓔ），それを死亡票によって補っている（ⓐ+ⓑ+ⓒ）．実際には届出漏れの生存例ⓓが存在するが，これを除いた合計（ⓐ+ⓑ+ⓒ+ⓔ）を罹患数とする．DCO割合とは，死亡票で初めて登録された患者（DCN；death certificate notification，図のⓐ+ⓑ）のうち，死亡診断書を発行した医療機関への確認調査（遡及調査）によって登録された患者数ⓑを除いた部分，すなわち死亡票の情報のみの患者数ⓐを罹患数（ⓐ+ⓑ+ⓒ+ⓔ）で割ったものである．

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(death certificate only)割合が用いられ,DCO割合が25%未満で精度が良いとされている(図1).

❷地域がん登録事業と個人情報保護のかかわり

地域がん登録事業は昭和57年に制定された老人保健法により,都道府県の行うべき事業と規定され(健医老第68号),罹患率,受療状況,生存率等の集計,解析によって,各地域のがんの動向を把握し,さらに市町村で実施されるがん検診事業の評価を行うこととなった.その後,平成15年5月に施行された健康増進法第16条により,「(生活習慣病の発生状況の把握)国及び地方公共団体は,生活習慣とがん,循環器病その他の生活習慣病との相関関係を明らかにするため,生活習慣病の発生状況の把握に努めること」,具体的な内容は,「地域がん登録事業及び脳卒中登録事業であること」として,一定の法的根拠を与えられるに至った.

地域がん登録では,ある地域で発生したがん症例を対象としているため,さまざまな医療機関から届出された情報をもとに同一の腫瘍かどうか,その個人を同定する必要がある.そのため個人識別情報(氏名,年齢,住所など)は必須である.そこで,プライバシー保護の観点から検討を重ね,平成8年に厚生省がん研究助成金「地域がん登録の精度向上と活用に関する研究」班(主任研究者:花井 彩)では,ガイドライン「地域がん登録における情報保護」を策定し,機密保持に十分に努めてきた.

平成14年6月には「疫学研究に関する倫理指針」(文部科学省・厚生労働省)により,個人情報を扱う場合にはインフォームド・コンセントが必要であることが示されたが,別添の「『疫学研究に関する倫理指針』とがん登録事業の取扱いについて」で「がん登録事業については,本指針は適用されない」と明記され,保健事業として従来どおり事業を実施することが可能となった.

しかし個人情報保護法,具体的には「個人情報の保護に関する法律(平成15年法律第57号)」,「行政機関の保有する個人情報の保護に関する法律(平成15年法律第58号)」および「独立行政法人等の保有する個人情報の保護に関する法律(平成15年法律第59号)」が平成15年5月に制定され,基本的に個人情報を扱う場合には対象者からインフォームド・コンセントを得ることが必須となった.

がん登録事業は,がん告知をされていない者や死亡した者からインフォームド・コンセントを得ることが難しいこと,また個人に不利益を及ぼすことがないこと,そして何よりも公衆衛生の推進のために必要な事業であることから,これらの法律の適用除外となることが望まれた.そのため,平成16年1月8日付けで厚生労働省健康局長通知(健習発第0108003号)が都道府県知事,政令市長,特別区長宛てに出され,「法令に従い個人情報の保護に十分な配慮をすることが重要であるが,地域がん登録事業の取扱いについては,利用目的による制限や第三者提供の制限の適用除外の事例に該当する」となった.なお本通知においても,前述のガイドライン「地域がん登録における情報保護」が別添として示されている.

●おわりに

地域がん登録事業は世界的にもがん罹患数の集計等に重要な役割を果たしている.このがん罹患数把握をさらに充実させるためにも,地域がん登録は院内がん登録と連携した形で今後,整備が進められることが必要である.また,地域がん登録事業はまだすべての都道府県で実施されているわけではなく,がん罹患数の全国値を完全に把握できているわけではない.がん罹患数を精巧かつ迅速性をもって把握するために,個人情報保護への対策も配慮した法的整備が求められている.

Clinical Section

Gerontology

Gerontology 2004;50:110–112
DOI: 10.1159/000075562

Received: February 17, 2003
Accepted: May 7, 2003

Effects of Dementia on Mortality in the Radiation Effects Research Foundation Adult Health Study

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Key Words
Alzheimer's disease · Vascular dementia · Mortality · Longitudinal study · Japan

Abstract
Background: Although dementia is rarely listed on death certificates, it does contribute to mortality. The predominant immediate causes of death coincident with dementia are pneumonia and cardiovascular diseases. ***Objective:*** To estimate the impact of dementia on specific mortality risks. ***Methods:*** We applied DSM-III/R criteria for Alzheimer's disease (AD) and vascular dementia (VaD) to 2,172 subjects of the Adult Health Study of the Radiation Effects Research Foundation who were 60 or more years old when examined from 1992 to 1996. The underlying causes of death were compiled from death certificates. We performed a Poisson regression analysis to evaluate specific causes of mortality for which AD or VaD was a significant risk factor. ***Results:*** The relative risk of mortality was 2.2 for AD and 2.4 for VaD. Mortality from pneumonia and stroke was elevated for both types of dementia, independent of other medical conditions. AD was also associated with death from trauma. ***Conclusion:*** Dementia was a predictor of death due to pneumonia, stroke, and trauma among the Japanese elderly. The prevention and early detection of those conditions are important in the medical care and treatment of dementia cases.

Introduction

Although relatively few death certificates report dementia [1, 2], it is a cause of increased mortality according to longitudinal studies in Europe [3, 4], the US [5], and Japan [6, 7]. Pneumonia and cardiovascular diseases are the predominant immediate causes of dementia-associated deaths [8–11]. Few reports evaluate the impact of dementia on specific disease risks. The purpose of this study was to estimate the impact of dementia on specific risks of mortality in the Radiation Effects Research Foundation (RERF) Adult Health Study (AHS) population. In the AHS, prevalence cases of dementia, based on the *Diagnostic and Statistical Manual of Mental Disorders*, 3rd edition, revised (DSM III/R) were identified between 1992 and 1996 [12]. The prevalence of dementia in the cohort was 7.2% and was thus similar to that in the general population [12]. Ascertainment of vital status in the cohort is essentially completed, and death certificates were consulted to determine the causes of death.

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Table 1. Study subjects by sex, age, and DSM III/R diagnosis

Sex	DSM III/R	Age				Total
		60–69	70–79	80–89	≥90	
Male	Non-dementia	371	132	84	8	595
	AD	1	2	7	3	13
	VaD	5	4	4	0	13
Female	Non-dementia	636	566	233	27	1,462
	AD	1	11	38	11	61
	VaD	3	10	12	3	28
Total		1,017	725	378	52	2,172

Table 2. RR and 95% CI derived from Poisson regression analysis

Cause of death	Total		Stroke		Ischemic heart disease		Pneumonia		Trauma	
	RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI
Sex (female/male)	0.44	0.36–0.55**	0.51	0.30–0.91*	0.50	0.23–1.21	0.26	0.14–0.49**	0.80	0.21–3.81
Age (5 year increments)	1.66	1.56–1.78**	1.61	1.37–1.92**	1.91	1.49–2.48**	1.96	1.60–2.43**	1.12	0.73–1.69
Medical history										
Heart disease	1.41	1.07–1.83*	0.84	0.35–1.75	4.16	1.85–9.10**	0.92	0.34–2.07	5.47	0.04–4.09
Stroke	1.48	1.05–2.06*	3.46	1.67–6.67**	1.28	0.27–4.08	0.64	0.18–1.76	5.37	0.90–23.7*
Cancer	1.47	1.06–2.00*	0.55	0.13–1.52	1.31	0.30–3.89	2.27	0.84–5.22	–	–
Dementia										
AD	2.17	1.52–3.06**	2.73	1.12–6.06*	1.54	0.34–4.97	5.90	2.62–12.9**	9.56	1.14–61.5*
VaD	2.38	1.41–3.87**	2.50	0.85–6.49	1.17	0.06–7.05	12.30	3.47–36.7**	2.49	0.12–21.9

* $p < 0.05$, ** $p < 0.01$.

Methods

The study subjects consisted of 2,172 subjects of the AHS in Hiroshima, 60 or more years old, who underwent screening for cognitive impairment between September 1992 and September 1996 [12]. The study was approved by the Human Investigation Committee at RERF, and all subjects gave informed consent before being examined. Based on DSM III/R criteria applied to cognitive fucntion tests, neurological examinations, and informant questionnaires, 74 cases of Alzheimer's disease (AD) and 41 of vascular dementia (VaD) were identified, while 2,057 subjects did not have dementia. Table 1 shows the study subjects by sex, age at baseline examination, and dementia diagnosis. The cohort was followed up until December 31, 1999, or until death if it occurred earlier. Deaths were confirmed by the Japanese family registration system. The causes of death were identified through death certificates and classified on the basis of the International Classification of Diseases (ICD). After adjusting for sex, age at baseline examination, and disease history (heart disease, stroke, and cancer), we performed Poisson regression analysis to determine whether AD or VaD was a significant risk factor for mortality from all causes, ischemic heart disease, stroke, pneumonia, and trauma. Disease histories were obtained from AHS health examinations that included history-taking, physical examination, and laboratory tests.

Results

During the follow-up period, 284 (13.8%) of the non-dementia cases, 43 (58.1%) of the AD cases, and 21 (51.2%) of the VaD cases died. The average follow-up periods for non-dementia, AD, and VaD were 5.2, 3.8, and 4.0 years, respectively. Among non-dementia cases, the predominant causes of death were cancer (33%), stroke (14%), pneumonia (9%), and ischemic heart disease (8%). In the combined AD and VaD cases, the predominant causes of death were pneumonia and stroke; 3 death certificates named dementia as the underlying cause of death. Among AD cases, the predominant causes of death were pneumonia (28%), stroke (19%), ischemic heart disease (7%), dementia (7%), trauma (5%), senility (5%), and others. Among VaD cases, the causes of death were stroke (29%), pneumonia (24%), senility (14%), ischemic heart disease (5%), trauma (5%), and others. Table 2 shows the results of Poisson regression analysis. Dementia increased the risk of mortality independently

of age at baseline, sex, and comorbidity. Relative risk (RR) of total mortality was 2.2 for AD and 2.4 for VaD. For both AD and VaD, mortality from pneumonia and stroke increased significantly. RR of mortality from stroke was 2.7 for AD and 2.5 for VaD, and RR of mortality from pneumonia was 5.9 for AD and 12.3 for VaD. AD also increased the risk of mortality from trauma significantly (RR = 9.6). There was no statistically significant difference of mortality from ischemic heart disease between dementia and non-dementia cases.

Discussion

This is one of the few population-based studies in which mortality associated with dementia has been investigated after adjustment for other medical conditions. Although dementia is rarely listed on death certificates [1, 2], dementia increases the risk of mortality significantly [3–5]. The RR of 2.2 for AD and 2.4 for VaD that we report here are close to those reported for community-based studies in the US and Europe, where the RR of mortality is 1.9–3.6 for dementia and 1.4–3.3 for AD [3–5]. Although dementia presents a significant risk for mortality, little is known about its impact on specific mortality risks. Burns et al. [9] and Keene et al. [11], using death certificate diagnoses, reported that the main immediate causes of death among subjects with dementia recorded at autopsy are pneumonia and cardiovascular disease, which is in agreement with our study. Among the AHS cohort, the RR of death from pneumonia was significantly higher for subjects with AD and VaD than for those without dementia, and it was higher than that of cancer. This result is compatible with the report by Morrison and Siu [10], who reported that patients with advanced dementia and pneumonia had a poor prognosis. In a previous study of the AHS population, the prevalence of VaD, but not AD, increased with a history of stroke [12], while in the present study, mortality from stroke increased not only in VaD but also in AD. Since many VaD patients had a history of stroke, RR of death from stroke among VaD patients may be close to the product of the risks.

Although stroke events among 7 AD patients occurred after the onset of dementia, the increased mortality from stroke in AD suggests that the etiology of AD may include a vascular component. Most demented subjects near the end of life have difficulty with the basic activities of daily living [1], and the increased mortality from trauma associated with AD might be caused by disabilities associated with dementia. Since RR of death from trauma among VaD patients is thought to be close to the product of the risks of stroke history and VaD, the increased mortality from trauma may be associated with VaD as well.

Increased mortality from pneumonia, stroke, and trauma was observed in demented subjects independent of other relevant medical conditions. Thus, the prevention and early detection of those events are necessary in the medical care and treatment of dementia cases.

Acknowledgements

This paper is based on research performed at the Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, Japan. RERF is a private non-profit foundation funded equally by the Japanese Ministry of Health, Labour and Welfare and the US Department of Energy through the National Academy of Sciences.

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Atherosclerosis 176 (2004) 91–94

ATHEROSCLEROSIS

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LTA 252G allele containing haplotype block is associated with high serum C-reactive protein levels

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Received 30 June 2003; received in revised form 13 November 2003; accepted 13 December 2003

Available online 4 July 2004

Abstract

C-reactive protein (CRP), an inflammatory biomarker, is a predictor of future risk for cardiovascular disease. Hypothetically, the levels of inflammatory response to microbial and lifestyle-related factors are influenced by genetic factors. LT-α is a proinflammatory cytokine that plays an important role in the pathogenesis of atherosclerosis in mice. We examined the association between gene polymorphism of the LT-α coding gene, *LTA* A252G, and CRP based on a case–control study. The top 149 and bottom 151 subjects in terms of CRP levels were selected for genotyping from among 1000 A-bomb survivors free from acute infection, chronic liver diseases, uremia, autoimmune diseases, and cancers. The genotype of *LTA* was determined by fluorescence resonance energy transfer–polymerase chain reaction (FRET–PCR) and subsequent melting curve analysis. The values of traditional risk factors such as body mass index (BMI), white blood cell (WBC) count, hemoglobin (Hb) concentration, and glycated Hb (HbA1c) differed significantly between the low and high CRP groups. After adjusting for the effect of sex, age, BMI, WBC, Hb, and HbA1c, the *LTA* 252G allele was found to be associated with high CRP levels (odds ratio = 1.93, $P = 0.007$) by multiple logistic regression analysis. Thus, CRP levels are influenced not only by environmental factors but also by the polymorphism of *LTA* or other genes in the same haplotype block.

Keywords: Inflammation; Interleukins; Risk factors; Atherosclerosis; Genetics

1. Introduction

Atherosclerosis is thought to be an inflammatory disease [1]. A number of studies have examined various circulation biomarkers of inflammation as potential predictors of future risk for cardiovascular disease. Among them, C-reactive protein (CRP) is one of the most consistent biomarkers in a variety of clinical settings of risk for cardiovascular disease [2–4]. In addition to microbial infection in arterial walls [5,6], traditional cardiovascular risk factors such as white blood cell (WBC) count, body mass index (BMI), age, and smoking were associated with high CRP in healthy individuals [7], indicating that these lifestyle-related factors also trigger low-grade inflammation. The biological significance of high CRP must be considered cautiously; however, it may reflect high levels of environmental stimuli that trigger inflammation, or possibly high responsiveness to such stimuli, possibilities that are not mutually exclusive.

LT-α (TNF-β) and TNF-α are proinflammatory cytokines coded for by the *LTA* and *TNFA* genes, respectively. Both cytokines induce apoptosis in cells upon binding to TNF receptor type 1, whereas they induce inflammatory responses by activating NFκB nuclear protein upon binding to TNF receptor type 2 [8]. Thus, the cytokines have the potential to boost inflammatory response. Moreover, both cytokines are expressed in atherosclerotic lesions [9,10]. A recent report indicates that atherosclerosis in atherogenic diet-fed mice was attenuated by the disruption of the *LTA* gene but not the *TNFA* gene [10]. This report prompted us to investigate the hypothesis that the atherosclerosis-related gene *LTA* in mice may influence serum CRP levels in healthy individuals. Here we report that the *LTA* 252G allele is associated with high CRP levels in healthy human subjects.

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0021-9150/$ – see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2003.12.013

2. Materials and methods

Subjects in this study were selected from among 1000 members of the Adult Health Study (AHS) longitudinal cohort established in 1958 who received medical examinations in Hiroshima from October 1999 to January 2001 and did not suffer from acute infection, chronic liver diseases, uremia, autoimmune diseases, or cancers. The AHS cohort is composed of four different radiation exposure categories, from zero- to high-dose groups [11]. Serum CRP levels were measured with a highly sensitive CRP-Latex kit using anti-CRP monoclonal antibodies (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). The top 149 and bottom 151 subjects in terms of serum CRP levels were selected for the present study. There was no difference between the two groups ($P = 0.68$) in the prevalence of cardiovascular disease (ICD code—ninth: 401–405, 410–414; tenth: I10–I15, I20–I25). Preliminary analyses demonstrated that radiation dose distribution did not significantly differ among low and high CRP groups ($P = 0.2644$ by Student's t-test) or three *LTA* genotype groups ($P = 0.0814$ by Student's t-test) in the present subjects. Thus, we did not include dose as a variable in the present analysis.

Written informed consent regarding genome research was obtained from the participants according to the Guidelines for Genome/Genetic Research issued by the Japanese government. RERF's Ethical Committee for Genome/Genetic Research approved this research protocol in our institute (pilot study no. B25).

Genomic DNA was extracted from whole blood using the QIAamp® DNA Blood Mini Kit (QIAGEN). The genotype of *LTA* was determined by fluorescence resonance energy transfer–polymerase chain reaction (FRET–PCR) technique with subsequent melting curve analysis using a real-time PCR instrument (Light Cycler®, Roche Diagnostics) [12]. PCR primers and fluorogenic probes used were: forward primer (5′-AGAGAAGGGGACAAGATGCAGT-3′), reverse primer (5′-GGCCTTGGTGGGTTTGGTT-3′), sensor probe (5′-CAGAGAGGAACCATGGCAGA-3′-FITC) and anchor probe (Red640-5′-CAGAGAATGTGTGACAGAGACAAT-3′). The thermocycling procedure consisted of 45 cycles of denaturation at 95 °C for 15 s, annealing at 65 °C for 15 s, and extension at 72 °C for 15 s using 3 mM $MgCl_2$. After amplification, a melting curve analysis was performed by changing the temperature from 95 °C for 1 s to 40 °C for 15 s, and then gradually raising the temperature to 70 °C over a period of 7 min. Accuracy of genotyping using the FRET–PCR method was validated in 21 cases by comparing the results with sequence data obtained by a PCR-based direct sequencing method.

2.1. Statistical analyses

Genotype frequencies of the subjects with high CRP levels were compared with those of the subjects with low CRP levels, after adjustment for potential confounders that included sex, age at sampling, BMI, WBC, Hb, HbA1c, total cholesterol, and smoking status. We computed the unadjusted and adjusted odds ratios (ORs) and their 95% confidence intervals (CIs) using multiple logistic regression models. The distribution of the *LTA* A252G genotype in the low CRP group corresponded well with the Hardy-Weinberg equilibrium ($P = 0.31$ by χ^2 statistics). All calculations were performed with STATA8 (Stata Corporation, College Station, TX).

3. Results

Basic characteristics of the high CRP and low CRP groups are shown in Table 1. Significant differences between the high and low CRP groups were observed in the levels of traditional risk factors for cardiovascular disease such as BMI, WBC, Hb, and HbA1c, but not in the distribution of sex, age, smoking, and total cholesterol. As mentioned in Section 2, radiation dose at the time of the A-bombings was not associated with either CRP or certain genotypes, and therefore we did not adjust for the dose in the following analyses.

Before adjustment for traditional cardiovascular risk factors, *LTA* 252G carriages were associated with high CRP (OR = 1.84, 95% CI = 1.13–3.02 for GA; OR = 2.08, 95% CI = 1.01–4.25 for GG genotype) (Table 2). After adjustment for traditional risk factors associated with high CRP (Table 2), the association remained between the *LTA* 252G allele and high CRP (OR = 1.93, 95% CI = 1.14–3.29), indicating that this positive association might not be affected by the traditional cardiovascular risk factors BMI, WBC, Hb, and HbA1c.

Table 1
Basic characteristics of subjects

	High CRP	Low CRP	P-value[a]
Sex			
Male (%)	62 (41.6%)	54 (35.8%)	0.30
Female (%)	87 (58.4%)	97 (64.2%)	
Cardiovascular disease	46[b] (30.9%)	50[b] (33.1%)	0.68
Smoking status			
No (%)	106 (71.1%)	109 (72.2%)	0.84
Yes (%)	43 (28.9%)	42 (27.8%)	
	Mean (S.D.)		
CRP (mg/dl)	0.254 (0.33)	0.009 (0.01)	<0.0001
Age (year)	59.8 (4.8)	59.2 (5.1)	0.26
BMI (kg/m^2)	24.4 (3.4)	22.0 (2.7)	<0.0001
WBC ($\times 10^2/\mu$l)	62.5 (18.0)	53.6 (13.3)	<0.0001
Hemoglobin (g/dl)	13.8 (1.4)	13.4 (1.2)	0.008
HbA1c (%)	5.7 (1.1)	5.2 (0.7)	0.0001
Total cholesterol (mg/dl)	218.7 (35.0)	220.6 (37.1)	0.66

S.D.: standard deviation.

[a] P-values from χ^2-test for group variables and P-values from unpaired t-test with unequal variance for continuous variables.

[b] Eight subjects each in the high and low CRP groups had coronary heart disease, and the others had hypertension.

Table 2
LTA 252G allele is dominantly associated with high CRP

	Low CRP no. (%)	High CRP no. (%)	Unadjusted			Adjusted		
			Odds ratio	95% CI	χ^2 (*P*-value)	Odds ratio[a]	95% CI	χ^2 (*P*-value)
Genotype								
AA	75 (49.7)	51 (34.2)	1.00		7.48	1.00		6.02
AG	59 (39.1)	74 (49.7)	1.84	(1.13–3.02)	(0.024)	1.94	(1.10–3.41)	(0.049)
GG	17 (11.3)	24 (16.1)	2.08	(1.01–4.25)		1.93	(0.85–4.37)	
Allele carriage								
AA	75 (49.7)	51 (34.2)	1.00		7.34	1.00		6.02
AG + GG	76 (50.3)	98 (65.8)	1.90	(1.19–3.02)	(0.007)	1.93	(1.14–3.29)	(0.014)

[a] Baseline odds were adjusted for sex, age, BMI, WBC, Hb, and HbA1c.

4. Discussion

Inflammation's key role in the pathogenesis of atherosclerosis is well established. Several possible mechanisms have been theorized: microbial infection in atherosclerotic plaque [5,6], inflammatory reaction caused by oxidative stress [13,14], and production of the inflammatory cytokine IL-6 by adipose tissue [15]. Thus, both microbes and lifestyle-related factors seem to trigger CRP production. Increasing evidence in different clinical situations has demonstrated that CRP is among the most consistent biomarkers for future risk of cardiovascular disease [2–4]. However, little is known about the effect of genetic factors on CRP in response to various stimuli.

Many polymorphic genes regulate inflammation and immune response. In this context, the TNF family of genes is of interest for three reasons. First, TNF-α and LT-α proteins are proinflammatory cytokines that are expressed in atherosclerotic lesions [9,10]. Second, the polymorphisms of *TNFA* and *LTA* are associated with levels of cytokine production and immune response [16]. Third, disruption of the *LTA* gene but not the *TNFA* gene attenuated atherosclerosis in atherogenic diet-fed mice [10]. Thus, *LTA* seems to play an important role in atherosclerosis, at least in mice. LT-α is a proinflammatory cytokine that induces inflammatory cytokines as well as acute-phase proteins such as CRP by inducing the nuclear factors NFκB and NF-IL6 [17,18]. In the present study, we clearly demonstrated a significant association between the *LTA* 252G allele and high CRP levels (Table 2). The result is concordant with Messer's report that the *LTA* 252G homozygote was associated with high LT-α production by phytohaemagglutinin-activated mononuclear cells [16]. The *LTA* 252G allele elevated CRP independent of the traditional cardiovascular risk factors BMI, WBC, Hb, and HbA1c (Table 2). Thus, these traditional risk factors might elevate CRP through molecular mechanisms other than LT-α induction.

Ozaki et al. recently reported an association between the *LTA* 252G allele and susceptibility to myocardial infarction [19]. The *LTA* 252G allele is completely linked to polymorphisms of two other genes in the Japanese population: the −63A allele of NFκB inhibitor-like 1 gene (*NFKBIL1*) and the −23C allele of *BAT119*. The −63A allele of *NFKBIL1* was reported to be associated with reduced transcriptional activity of the gene [19]. The physiological significance of *NFKBIL1* polymorphism in vivo is not clear, it might potentially affect inflammatory response through altering the translocation of nuclear protein NFκB from cytoplasm to nucleus. *BAT1* is a member of the DEAD-box family of RNA-dependent ATPases. Inactivation of *BAT1* resulted in the higher production of proinflammatory cytokines in cultured cells [20], indicating a negative regulatory role for *BAT1*. Since the −23C allele of *BAT1* was suspected to reduce gene expression [21], it might affect the levels of proinflammatory cytokines in vivo. Although *NFKBIL1* −63A, *BAT1* −23C and *LTA* 252G alleles in the same haplotype block may potentially affect the levels of CRP, it is premature to conclude which gene polymorphism is dominant in terms of increased CRP levels. Future studies will elucidate the specific gene polymorphism that affects CRP levels.

In the present study, CRP was not associated with the prevalence of cardiovascular disease, although CRP is a predictor for future risk of coronary heart disease. In Japan, the prevalence of myocardial infarction was low compared with that in the US [22]. Most subjects with cardiovascular disease in the present study were those with hypertension, and only eight subjects each in the low and high CRP groups had coronary heart disease. Those numbers were too small to demonstrate an association between CRP and coronary heart disease.

The subjects in the present study were selected from the cohort of atomic bomb survivors. There is a concern that the cohort might be biased in terms of survivability in an atomic bombing disaster. We have to be cautious about whether gene polymorphisms potentially associated with survivability are overly represented in A-bomb survivors. Our cohort consists of subjects with different radiation exposure categories, from zero to high dose, which enables us to assess the association between dose and genotype. As mentioned in Section 2, there was no significant association between *LTA* genotype and radiation dose. Thus, we believe that the present results are not biased in the selection of samples.

This study is the first to demonstrate that the levels of CRP in healthy individuals are influenced not only by

environmental factors but also by a genetic factor, i.e. the polymorphism of *LTA* or closely linked genes.

Acknowledgements

This publication is based on research performed at the Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, Japan. RERF is a private nonprofit foundation funded equally by the Japanese Ministry of Health, Labour and Welfare and the US Department of Energy through the US National Academy of Sciences. Additional funding for this project was provided by grant number 14657109 from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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RADIATION RESEARCH 161, 642–647 (2004)
0033-7587/04 $15.00

An Association between Oxidative Stress and Radiation-Induced Lymphomagenesis

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Suzuki, G., Shimada, Y., Hayashi, T., Akashi, M., Hirama, T. and Kusunoki, Y. An Association between Oxidative Stress and Radiation-Induced Lymphomagenesis. *Radiat. Res.* 161, 642–647 (2004).

It is generally thought that reactive oxygen species (ROS) play an important role in carcinogenesis. However, direct evidence supporting this idea is still lacking. In the present study, we measured ROS in thymocytes at the thymic prelymphoma stage in C57BL/6 mice. Mice (n = 20) were irradiated at 1.6 Gy/week for 4 consecutive weeks and the levels of ROS were measured 8 to 11 weeks later by dehydrorhodamine 123, which accumulated in mitochondria and became fluorescent dye upon oxidation. Unirradiated littermates (n = 17) served as controls. Thymic prelymphoma cells were diagnosed by the aberrant CD4/CD8 staining profile and monoclonal or oligoclonal T-cell receptor gene rearrangement. A significant fraction of mice (11/13) bearing thymic prelymphoma cells exhibited elevated levels of ROS in thymocytes ($P < 0.001$). The result is consistent with the hypothesis that ROS may play an important role in radiation carcinogenesis. © 2004 by Radiation Research Society

INTRODUCTION

There is increasing evidence that most cancers contain multiple mutations, and the accumulation of dysfunction in several key molecules seems essential for carcinogenesis (*1*). In normal cells, the mutation rate is low, of the order of 10^{-10} mutations per nucleotide per cell per generation, which is insufficient to account for the large numbers of mutations observed in cancer cells (*2*). It is believed that only cells with mutator phenotypes or genomic instability will accumulate genetic alterations required for carcinogenesis. Radiation is a well-known mutagen that induces many kinds of neoplasms (*3*). Radiation can acutely induce DNA double-strand breaks, single-strand breaks, and crosslinking between DNA and protein in a dose-dependent manner (*4*). The impact of these alterations on cells may either initiate the process of multistage carcinogenesis in stem cells or accelerate the process already initiated in cells by other mutagens. Furthermore, radiation is believed to have a long-lasting effect on the progeny of irradiated cells by inducing genomic instability, and it may shorten the period required for the progression of each step in multistage carcinogenesis (*5–7*). However, the molecular mechanisms of induction of genomic instability by radiation have not been fully elucidated.

Reactive oxygen species (ROS) are generated constantly in cells and injure DNA in the nucleus as well as deoxynucleotide triphosphate in its cellular pool. Y-family polymerases erroneously incorporate an oxidized deoxynucleotide into DNA or repair an oxidized DNA erroneously (*8, 9*). Thus an increase in the production of ROS may cause genomic instability and facilitate the process of multistage carcinogenesis. In support of this idea, mice with a mutation in the cytochrome b subunit exhibited an increase in ROS production and a high mutation rate.[2] Recently, it became evident that radiation elevates ROS in the progeny of irradiated cells *in vitro* (*10–12*), which forced us to test the hypothesis that one of the mechanisms of radiation-induced genomic instability might be the prolonged elevation of ROS *in vivo*. To test this, we took advantage of a radiation-induced thymic lymphoma model in which more than 90% of irradiated B10 mice generated thymic lymphoma (*13*), and thymic prelymphoma cells could be detected by the presence of oligoclones having a particular T-cell receptor (TCR) gene rearrangement in the thymus. ROS were measured directly in mitochondria by the oxidation-sensitive fluorescent probe dihydrorhodamine 123 (DHR) (*10*). Our results clearly demonstrated significant associations of elevated oxidative stress with aberrant CD4/CD8 staining profile and mono- or oligoclonal TCR gene rearrangement in thymocytes at the thymic prelymphoma stage in irradiated mice. This information supported the hypothesis that an increase in generation of ROS might be one of the mech-

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anisms of radiation-induced genomic instability that could lead to lymphomagenesis.

MATERIALS AND METHODS

Mice

C57BL/6 mice were bred and kept in our conventional animal facility in the National Institute of Radiological Sciences, Chiba. Four-week-old female mice were X-irradiated with 1.6 Gy four times for 4 consecutive weeks using a Pantak X irradiator (Pantak Ltd., East Haven, CT) at 200 kVp, 20 mA with 0.5 mm copper and aluminum filters. Unirradiated female littermates served as controls. Several weeks after irradiation, mice were transferred to the Radiation Effects Research Foundation, Hiroshima, and kept in a conventional animal facility. Three to five irradiated mice and unirradiated littermates were killed humanely 8 to 11 weeks after the last irradiation, as indicated. The chairman of the institutional animal use committee reviewed and assured that all experiments had been done following the International Guiding Principles for Biomedical Research Involving Animals issued by WHO in 1985.

Flow Cytometry Analyses

Thymocytes (1×10^6) were suspended in 1 ml of RPMI-1640 medium supplemented with 2% fetal bovine serum. Cells were stored in the dark and incubated with DHR (Molecular Probes, Eugene, OR) at a final concentration of 1 μM at 37°C for 80 min. DHR is a pro-fluorescent dye that accumulates in mitochondria and becomes fluorescent rhodamine-123 upon oxidation by ROS. After washing once with medium, cells were analyzed by flow cytometry (FACScan Becton Dickinson, Mountain View, CA). The mean fluorescence intensity was calculated using CellQuest software (Becton Dickinson). Thymocytes were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 and analyzed with the FACScan. In the case of three-color staining, cells incubated with DHR were further reacted with CyChrome-conjugated anti-CD4 and PE-conjugated anti-CD8. All fluorochrome-labeled reagents were purchased from PharMingen (San Diego, CA).

TCR Gene Rearrangement

Genomic DNA was extracted from thymocytes using Qiagen Genomic-tip 100 (Qiagen, Tokyo). Specific primers and PCR conditions for the amplification of the genomic DNA segment containing TCR Dβ and Jβ genes were reported elsewhere (*14*). By combining Dβ sense primer and Jβ anti-sense primer, i.e. Dβ1-Jβ1.5, Dβ1-Jβ1.6, Dβ1-Jβ2.6 and Dβ2-Jβ2.6, every TCR Dβ-Jβ rearrangement band could be detected (*14*).

Statistical Analyses

Fisher's exact probability test was used.

RESULTS AND DISCUSSION

More than 90% of the irradiated mice developed thymic lymphoma after an average latent period of 200 days in B10 mice (*13*). Since some mice developed thymic lymphoma as early as 8 weeks, we performed flow cytometry analyses 8 to 11 weeks after irradiation. As shown in Table 1, only 2 of 20 irradiated mice bore overt thymic lymphoma, and 7 showed a decreased number of thymocytes (Table 1). Normal thymocytes are composed of four subpopulations, $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), $CD4^+CD8^-$ single positive (SP), and $CD4^-CD8^+$ SP cells. DN cells are the most immature cells

TABLE 1
Effect of Radiation on the Regeneration of Thymocytes

Weeks after irradiation	Cell count[a] ($\times 10^8$)	CD4/CD8 staining profile	Elevation in ROS[b]
8	4.2*[c]	Not done	no
8	0.5*	Not done	yes (1.7)
8	1.2	Not done	no
9	0.6*	normal	yes (1.7)
9	1.9	normal	yes (1.5)
9	6*[c]	DP/CD8	no
9	0.14*	CD8	yes (2.5)
9	2.5*	DP/CD8	no
9	0.16*	DP/CD8	yes (3.3)
9	1.7	DP/CD8	yes (1.5)
10	1.3	normal	no
10	1.2	DP	yes (1.3)
10	0.58*	DP/CD8	yes (1.9)
10	2.4	CD8	yes (1.7)
10	2.3	CD8	yes (2.1)
11 (no. 6)[d]	2.3	CDP/CD8	yes (1.5)
11 (no. 7)[d]	1.7	normal	no
11 (no. 8)[d]	0.53*	DP/CD8	yes (2.2)
11 (no. 9)[d]	1.8	DP	yes (1.4)
11 (no. 10)[d]	0.55*	DP	yes (1.6)

[a] Seventeen littermates served as controls. Thymocyte counts in control mice were $1.65 \pm 0.43 \times 10^8$ ($n = 17$). *Those cell counts were more or less than 2 SD away from the thymocyte counts in the control mice.

[b] Elevation of ROS was determined if the mean fluorescence intensity of DHR staining exceeded more than 3 SD of the control mice. The elevation in ROS was measured as the ratio between the mean fluorescence intensity of each experimental mouse and the average mean fluorescence intensity of the control mice.

[c] These two mice were diagnosed as having thymic lymphoma.

[d] These five mice were tested for TCRβ gene rearrangement. Numbers in parentheses are numbers of the mice in the experiment in Fig. 2.

that differentiate into DP cells through intermediate CD8 SP cells. Only a small fraction of DP cells differentiate into either CD4 or CD8 SP cells after so-called thymic selection, and these SP cells immigrate to peripheral lymphoid organs (*15, 16*). Figure 1A shows the representative staining profiles of these four thymocyte subpopulations in normal thymus. In contrast to control mice, 13 of 17 irradiated mice showed aberrant staining profiles (Table 1), in which CD8 SP and/or DP cells increased independent of thymocyte cell numbers (Fig. 1B–D).

Next we measured the levels of ROS in mitochondria in thymocytes by DHR staining. In each experiment, the reference level of ROS in mitochondria was determined in three to five control mice, and the mean fluorescence intensity of DHR staining was calculated. We judged a mouse to have elevated levels of ROS if the mean fluorescence intensity of DHR staining was more than the mean plus 3 SD of control mice. The levels of ROS in normal mice were fairly constant from mouse to mouse (Fig. 1E). In contrast, 14 of 20 irradiated mice showed an increase in ROS in mitochondria (Table 1). Representative DHR staining profiles are shown in the lower panel of Fig. 1. The difference in DHR staining levels was not due solely to the difference

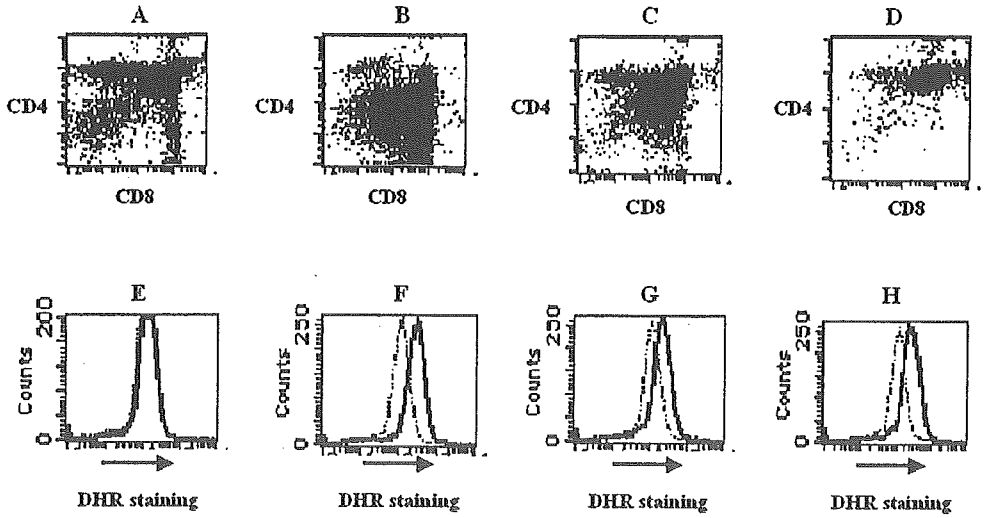


FIG. 1. Irradiated thymus bears an aberrant T-cell subpopulation with elevated ROS in mitochondria. Representative double-staining profiles of CD4 and CD8 are shown. Panel A: normal mouse; panel B: aberrant increase in $CD8^+$ cells; panel C: aberrant increase in $CD8^+$/DP cells; panel D: aberrant increase in DP cells; panel E: the overlay histograms of DHR staining profile in five control mice; panels F, G, H: DHR staining profile of an irradiated mouse (solid line) overlaid with that of a control mouse (dotted line). Thymocytes in panels B and panels F, C and G, and panels D and H were from the same individual mice.

in cell size between irradiated and normal mice; when the same cell-sized thymocytes as determined by forward scatter size in the flow cytometry analysis were compared, the levels of DHR staining were brighter in thymocytes from irradiated mice than in those from control mice.

The association between the CD4/CD8 staining profile and ROS levels was analyzed in 17 irradiated and 17 control mice. Eleven of 13 mice with an aberrant CD4/CD8 staining profile exhibited increased levels of ROS while only 2 of 21 mice with normal CD4/CD8 staining did (Table 2). Thus an aberrant CD4/CD8 staining status was significantly associated with an increase in ROS in mitochondria ($P < 0.001$).

Next we assessed whether elevated levels of ROS were associated with the clonal expansion of thymic prelymphoma cells that showed an aberrant CD4/CD8 staining profile in five control and five irradiated mice by PCR. Mature T cells bear heterodimeric receptors: either TCRαβ or TCRγδ. In the thymus, T cells sequentially rearrange the TCR-Vβ, -Dβ, and -Jβ genes at the DN stage and the TCRα gene at the DP stage. Using four combinations of Dβ-sense and Jβ anti-sense primers, all possible combinations of TCR Dβ-Jβ gene rearrangements were detected as different PCR bands of almost the same intensity in normal thymocytes (Fig. 2, mouse nos. 1–5). In contrast, four of five irradiated mice showed monoclonal or oligoclonal bands; Dβ1-Jβ2.4 band in no. 6, Dβ1-Jβ2.5 band in no. 8, Dβ1-Jβ1.5, Dβ2-Jβ2.2, Dβ1-Jβ2.5 and germ-line bands in no. 9, Dβ1-Jβ1.4, Dβ2-Jβ2.2, Dβ2-Jβ2.3 and germ-line bands in no. 10 (Fig. 2A–D). As indicated in Table 1, these four mice showed aberrant CD4/CD8 profiles and elevated ROS, while mouse no. 7 showed a normal CD4/CD8 staining profile and reference levels of ROS. There was a significant association between elevated ROS and mono- or oligoclonal TCRβ gene rearrangement (Table 3). Collectively, these data indicated that monoclonal or oligoclonal expansion of thymic prelymphoma cells was associated with aberrant CD4/CD8 staining and an increase in ROS in mitochondria.

Radiation acutely generates both short-lived and long-

TABLE 2
Association between Elevated ROS and Aberrant CD4/CD8 Staining Profile

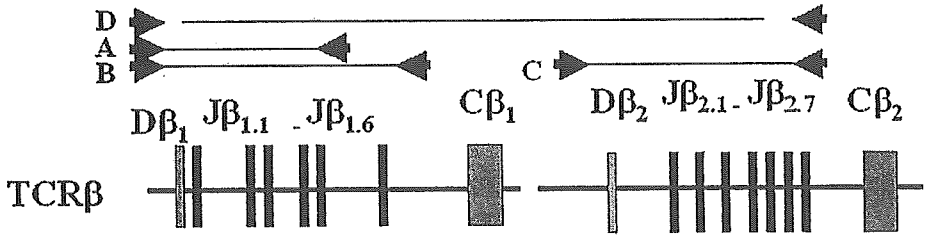
	CD4/CD8 staining profile[a]		
	Normal	Aberrant	Total
Normal ROS level	19	2	21
Elevated ROS level	2	11[b]	13
Total	21	13	34

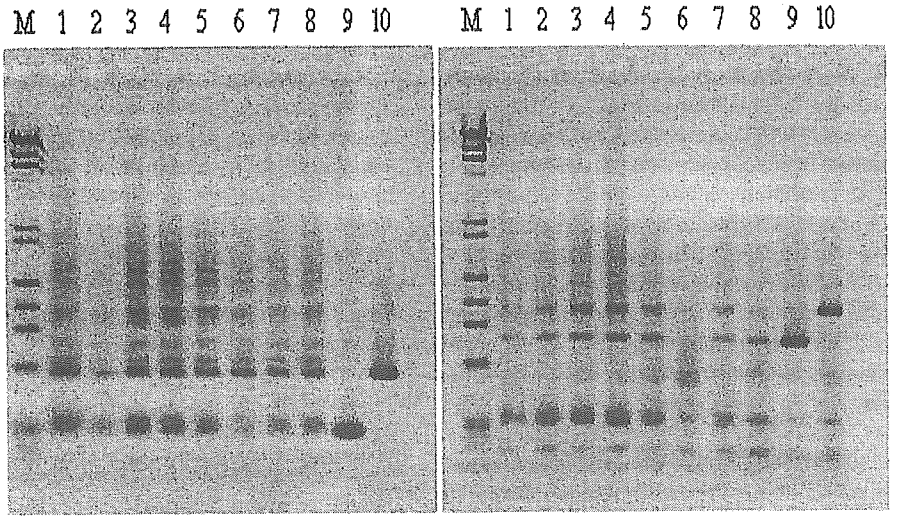
[a] Seventeen irradiated and 17 control mice were examined for CD4/CD8 staining and ROS levels.

[b] Statistically significant by Fisher's exact probability test ($P = 0.000018$).

→

FIG. 2. Mono- or oligoclonal TCRβ gene rearrangement is detected in thymic prelymphoma cells. Schematic presentation of TCR Dβ and Jβ gene segments and four primer sets used for PCR: panel A: Dβ1-Jβ1.5; panel B: Dβ1-Jβ1.6; panel C: Dβ2-Jβ2.6; panel D: Dβ1-Jβ2.6 (upper row). PCR was performed using genomic DNA of individual thymus from normal (lanes 1 to 5) and irradiated (lanes 6 to 10) mice. The marker lane was the mixture of λDNA digested by *Hin*dIII and φX174 DNA digested by *Hae*III.


D
A
B
C
Dβ1 Jβ1.1 - Jβ1.6
Cβ1
Dβ2 Jβ2.1 - Jβ2.7
Cβ2
TCRβ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M 1 2 3 4 5 6 7 8 9 10

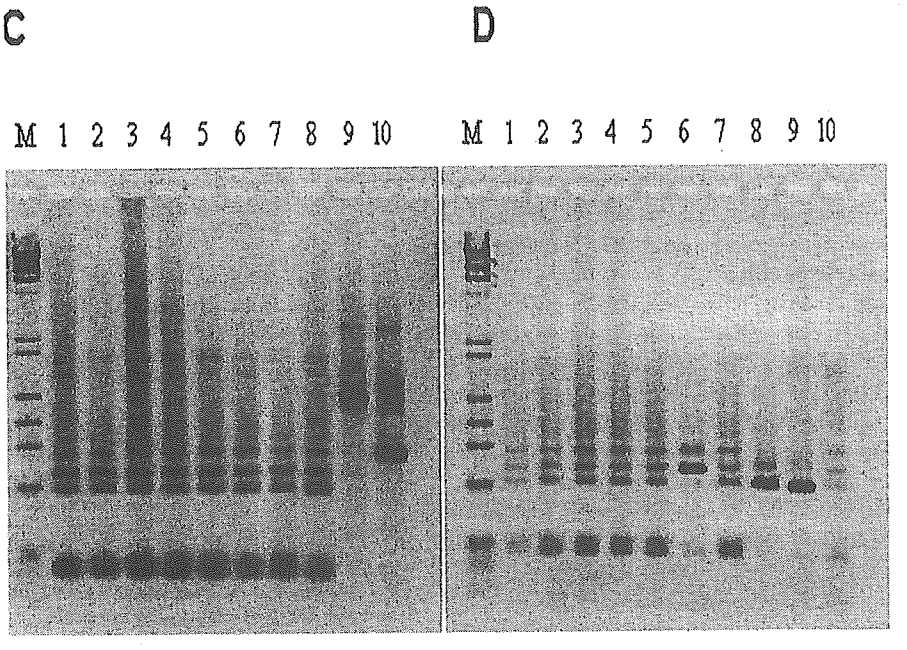
C
D
M 1 2 3 4 5 6 7 8 9 10
M 1 2 3 4 5 6 7 8 9 10

TABLE 3
Association between Elevated ROS Levels and Mono- and Oligoclonal TCRβ Gene Rearrangement

	TCRβ gene rearrangement[a]		
	Polyclonal	Mono- or oligoclonal	Total
Normal ROS	6	0	6
Elevated ROS	0	4[b]	4
Total	6	4	10

[a] Five irradiated and five control mice were examined for TCRβ gene rearrangement. Clonality was judged by a pattern of TCR Dβ-Jβ gene rearrangement shown in Fig. 2.

[b] Statistically significant by Fisher's exact probability test (P = 0.0048).

lived radicals (*17, 18*). Half-lives of radicals are generally short, and even a long-lived sulfinyl radical has a half-life of 20 h (*17*). However, Clutton *et al.* first reported that the progeny of irradiated bone marrow cells in culture exhibited an increase in production of ROS even 7 days after irradiation and proposed a hypothesis that enhanced and persistent oxidative stress might cause genomic instability after irradiation (*10*). Recently, two other groups confirmed the observation of Clutton *et al.* using normal fibroblasts or cell lines (*11, 12*). Since generation of ROS *in vitro* might be influenced by culture conditions such as confluence (*11*), it is important to investigate whether the progeny of irradiated stem cells exhibit an increase in production of ROS *in vivo*. In the present study we provided evidence that the hypothesis of Clutton *et al.* may also be plausible *in vivo*.

It was noted that the elevation of ROS was not completely associated with thymic prelymphomas or lymphomas; two of the irradiated mice with normal CD4/CD8 staining profiles exhibited elevated ROS levels, while three irradiated mice with either lymphoma or an aberrant CD4/CD8 staining profile showed normal levels of ROS. The results imply that ROS may be important but not essential for the whole process of lymphomagenesis. Once the critical stage for lymphomagenesis has passed, ROS may no longer be required, and in some mice, no elevation was observed when mice were killed.

The mechanisms resulting in an increase in ROS in mitochondria in thymic prelymphoma cells were beyond the scope of the present study. The following, however, are plausible mechanisms. First, DNA damage might generate ROS by activating Trp53 (*19, 20*). Limoli reported that chromosomally unstable cells showed elevated levels of ROS (*21*). In the case of immature T cells, DN and DP cells make endogenous DNA double-strand breaks by RAG1/RAG2 recombinase in the process of TCR V(D)J gene rearrangement. The levels of DHR staining were 2.2-fold higher in DN and DP cells than those in CD4 and CD8 SP cells in normal mice (data not shown). However, the levels of DHR staining in thymic prelymphoma cells were higher than those in normal thymocytes, 85% of which were DP cells, indicating that activation of RAG1/RAG2 recombinase was insufficient to elevate ROS to the levels observed in thymic prelymphoma cells. Second, the increase in production of ROS might be caused by mutations of genomic genes that affect the function of either mitochondrial respiratory chains or cellular redox proteins (*22, 23*). Third, radiation might affect mitochondrial DNA, which plays a role in the respiratory chain (*22*). Although there are hundreds to thousands of mitochondria in one cell, mitochondria affected by mutagens can be distributed unevenly into daughter cells and can accumulate sufficiently to elevate ROS levels in the progeny (*22, 24*). It was also reported that the accumulation of mitochondria lacking respiratory function disrupted the pro-oxidant/antioxidant balance in cells and thereby elevated levels of ROS (*25*). Fourth, radiation might have affected stromal cells in the thymus so as to produce excess amounts of bioactive substances such as chemokines or proinflammatory cytokines capable of up-regulating ROS levels in thymocytes. Further studies are needed to investigate the mechanisms of elevated ROS levels in thymic prelymphoma cells.

Finally, the present study clearly demonstrated an association between ROS and lymphomagenesis, suggesting that mitochondria play an important role in multistage carcinogenesis.

ACKNOWLEDGMENTS

The Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, Japan, is a private nonprofit foundation funded by the Japanese Ministry of Health, Labour and Welfare (MHLW) and the U.S. Department of Energy (DOE), with the latter provided through the U.S. National Academy of Sciences.

Received: January 21, 2004; accepted: February 20, 2004

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