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Discussion

Multiple papers have reported improvement in efficiency of RNA extraction from buffered formalin-fixed, paraffin-embedded tissue. Few reports, however, have reported on elimination of modification induced by buffered formalin-fixation, although RT-PCR amplification of RNA extracted from archival formalin-fixed, paraffin-embedded tissue was hindered by not only degradation of RNA but also modification of RNA bases by formalin. Preheating of RNA in TE buffer (pH 7.0) restored the template activity of RNA extracted from buffered formalin-fixed tissue where clear bands of 18S and 28S ribosomal RNA were still detected with partial degradation (Masuda et al. 1999). The reaction between formaldehyde and nucleotide monomers takes place in two steps. Primary reaction is to form labile methylol-derivatives by addition of formaldehyde group to NH-group of bases. Secondary slow reaction is to give rise to stable methylene derivatives in only amino purines (Feldman 1973; Auerbach et al. 1977). The reaction between formaldehyde and RNA is also thought to occur in the same manner. In fact, MALDI-TOF analysis has indicated that all four bases of RNA treated with buffered formalin were modified mainly by addition of mono-methylol groups (Masuda et al. 1999). Modification of nucleotides by addition of methyol groups is a reversible reaction: heating of RNA with 10 mM TE buffer (pH 7.0) at 70C results in removal of methylol derivatives from bases (Masuda et al. 1999).

Our results indicate that efficiency of RT-PCR amplification with degraded RNA extracted from long-term preserved unbuffered formalin-fixed, paraffin-embedded tissue specimens (for 19- 21 years) is improved by the heating of RNA in citrate buffer prior to cDNA synthesis. This enhanced efficiency was possibly caused by RNA modification elimination and subsequent RNA template activity restoration.

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Fragment sizes of around 60 bp can be amplified successfully at a rate of about 80% by RT-PCR, even when using RNA extracted from archival formalin-fixed, paraffin-embedded tissue samples stored for a long time. However, as in the case of amplification of the *N-ras* gene in two samples used in this study, there still remain archival tissue samples in which a weak band is only vaguely observed or not detected even when fragment size by RT-PCR amplification is around 60 bp.

Furthermore, when amplicon size is very small, it is often difficult to design primers in restricted regions such as fusion points. Therefore, increased efficiency of RT-PCR amplification by the preheating of RNA is most effective when only limited quantities of archival formalin-fixed, paraffin-embedded tissue samples are available for study or when the designing of primers in restricted regions cannot be avoided.

Heat treatment with alkaline solution (pH 9 - 12) for DNA extraction from archival formalin-fixed, paraffin-embedded tissue increased the efficiency of DNA extraction, resulting in enhanced PCR amplification (Shi et al. 2002, 2004). Our results demonstrate that heat treatment in citrate buffer with pH ranging from 3 to 6.5 improves to some extent the efficiency of RT-PCR amplification of RNA extracted from archival formalin-fixed, paraffin-embedded tissue, whereas treatment of RNA with pH solution ranging from 9 to 10 reduced the efficiency of RT-PCR amplification. These findings indicate that RNA or DNA modification induced by formalin may be removed more efficiently by preheating in acidic or alkaline buffer compared with neutralized buffer. The efficiency of RT-PCR amplification enhanced by RNA preheating in citrate buffer (pH 3 - 6.5) may be due to the fact that RNA is relatively stable in weak acidic solution but unstable in alkaline solution.

Treatment with highly concentrated citrate buffer reduced the efficiency of RT-PCR

amplification of RNA compared with non-treated RNA. In our experiment, incubation time of 30 - 60 min in citrate buffer with pH 4.0 was the most efficient method for RT-PCR amplification. A longer incubation time, such as 2 hr, resulted in slightly decreased efficiency of RT-PCR amplification, suggesting that degradation of RNA may occur to some extent during the long preheating in citrate buffer at 70C.

Preheating of RNA in citrate buffer resulted in improved efficiency of RT-PCR amplification in all five archival tissue specimens examined, suggesting that this method is useful for molecular analyses of long-term preserved tissue specimens. This technique will enable the qualitative analysis such as DNA rearrangement using degraded RNA extracted from archival unbuffered formalin-fixed, paraffin-embedded tissue specimens that have been stored for more than several decades. It will cast the light on the retrospective studies of rare cancers or cancers associated with exposure to uncommon past events, such as Thorotrast treatment, nuclear power station accidents, or atomic bombings.

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Literature Cited

Auerbach C, Moutschen-Dahmen M, Moutschen J (1977) Genetic and cytogenetical effects of formaldehyde and related compounds. Mutat Res 39: 317-362

Bresters D, Cuypers HTM, Reesink HW, Chamuleau RAFM, Schipper MEI, Boeser-Nunnink BDM, Lelie PN, Jansen PLM (1992) Detection of hepatitis C viral RNA sequences in fresh and paraffin-embedded liver biopsy specimens of non-A, non-B hepatitis patients. J Hepatol 15: 391-395

Bresters D, Schipper MEI, Reesink HW, Boeser-Nunnink BDM, Cuypers HTM (1994) The duration of fixation influences the yield of HCV cDNA-PCR products from formalin-embedded liver tissue. J Virol Methods 48: 267-272

Byers R, Roebuck J, Sakhinia E, Hoyland J (2004) PolyA PCR amplification of cDNA from RNA from formalin-fixed paraffin-embedded tissue. Diagn Mol Pathol 13: 144-150

Cohen CD, Gröne H-J, Gröne EF, Nelson P, Schlöndorff D, Kretzler M (2002) Laser microdissection and gene expression analysis on formaldehyde-fixed archival tissue. Kidney International 61: 125-132

Cronin M, Pho M, Dutta D, Stephans JC, Shak S, Kiefer MC, Esteban JM, Baker JB (2004) Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. Am J Pathol 164: 35-42

Feldman MY (1973) Reactions of nucleic acids and nucleoproteins with formaldehyde. Prog Nucleic Acids Res Mol Biol 13: 1-49

Finke J, Fritzen R, Ternes P, Lange W, Dölken (1993) An improved strategy and a useful housekeeping gene for RNA analysis from formalin-fixed, paraffin-embedded tissues by PCR. Biotechniques 14: 448-453

Forsthoefel KF, Papp AC, Snyder PJ, Prior TW (1992) Optimization of DNA extraction from formalin-fixed tissue and its clinical application in Duchenne muscular dystrophy. Am J Clin Pathol 98: 98-104

Frank TS, Svoboda-Newman SM, His ED (1996) Comparison of methods for extracting DNA from formalin-fixed paraffin sections for nonisotopic PCR. Diagn Mol Pathol 5: 220-224

Goldsworthy SM, Stockton PS, Trempus CS, Foley JF, Maronpot RR (1999) Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. Mol Carcinogenesis 25: 86-91

Jackson DP, Lewis FA, Taylor GR, Boylston AW, Quirke P (1990) Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. J Clin Pathol 43: 499-504

Kim J-O, Kim H-N, Hwang M-H, Shin H-I, Kim S-Y, Park R-W, Park E-Y, Kim I-S, van Wijnen AJ, Stein JL, Lian JB, Stein GS, Choi J-Y (2003) Differential gene expression analysis using paraffin-embedded tissues after laser microdissection. J Cell Biochem 90: 998-1006

Koopmans M, Monroe S, Coffield LM, Zaki SR (1993) Optimization of extraction and PCR amplification of RNA extracts from paraffin-embedded tissue in different fixatives. J Virol Methods 43: 189-204

Körbler T, Gršković M, Dominis M, Antica M (2003) A simple method for RNA isolation from formalin-fixed and paraffin-embedded lymphatic tissues. Exp Mol Pathol 74: 336-340

Lehmann U, Kreipe H (2001) Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embeded biopsies. Methods 25: 409-418

Masuda N, Ohnishi T, kawamoto S, Monden M, Okubo K (1999) Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res 27: 4436-4443

Mies C (1994) Molecular biological analysis of paraffin-embedded tissues. Hum Pathol 25: 550-560

Oliver KR, Heavens RP, Sirinathsinghji DJS (1997) Quantitative comparison of pretreatment regimens used to sensitize in situ hybridization using oligonucleotide probes on paraffin-embedded tissue. J Histochem Cytochem 45: 1707-1713

Park YN, Abe K, Li H, Hsuih T, Thung SN, Zhang DY (1996) Detection of hepatitis C virus RNA using ligation-dependnt polymerase chain reaction in formalin-fixed, paraffin-embedded liver tissues. Am J Pathol 149: 1485-1491

Rupp GM, Locker J (1988) Purification and analysis of RNA from paraffin-embedded tissues. Biotechniques 6: 56-60

Shi S-R, Cote RJ, Wu L, Liu C, Datar R, Shi Y, Liu D, Lim H, Taylor CR (2002) DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: Heating under the influence of pH. The J Histochem Cytochem 50: 1005-1011

Shi S-R, Datar R, Liu C, Wu L, Zhang Z, Cote RJ, Talor CR (2004) DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution. Histochem Cell Biol 122: 211-218

Sibony M, Commo F, Callard P, Gasc J-M (1995) Enhancement of mRNA *in situ* hybridization signal by microwave heating. Lab Invest 73: 586-591

Specht K, Richter T, Müller U, Walch A expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am J Pathol 158: 419-429

Weizäcker FV, Labeit S, Koch HK, Oehlert W, Gerok W, Blum HE (1991) A simple and rapid

JHC ex*PRESS*
Online publication date March 6, 2006
This article may be cited as DOI: 10.1369/jhc.5A6859.2006

method for the detection of RNA in formalin-fixed, paraffin-embedded tissues by PCR amplification. Biochem Biophys Commun 174:176-180, Werner M, Höfler H (2001) Quantitative gene

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Figure legends

Figure 1. Total RNA extracted from archival unbuffered formalin-fixed, paraffin-embedded thyroid cancer tissue. The extracted RNA was electrophoresed on 3% native agarose gel. Lanes 2-4 contain RNA from three different archival samples. Lane 1: 100-nucleotide RNA marker, lanes 5-6: 70-base synthesized nucleotides (25 ng and 100 ng/lane, respectively).

Figure 2. Effect of preheating temperature on RNA integrity. Two μg of total RNA extracted from frozen human thyroid cancer cell line were heated for 15 min or 30 min at various temperatures (lanes 4 and 5: 60C, lanes 6 and 7: 65C, lanes 8 and 9: 70C, lanes 10 and 11: 75C, lanes 12 and 13: 80C, lanes 14 and 15: 85C, lanes 16 and 17: 90C, lanes 18 and 19: 95C). Lane 3: no heating, lanes 1: λ *Hind* III DNA marker, lane 2: pUC19-*Msp*I digest for DNA size marker. Electrophoresis was done on 1.5% native agarose gel. Fifteen min-heating is for lanes 4, 6, 8, 10, 12, 14, 16 and 18. Thirty min-heating is for lanes 5, 7, 9, 11, 13, 15, 17 and 19.

Figure 3. Effect of pH on RT-PCR amplification of RNA. Efficiency of RT-PCR amplification of RNA preheated at 70C for 30 min in various pHs (lane 3: pH 3.0, lane 4: pH 4.0, lane 5: pH 5.0, lane 6: pH 6.0, lane 7: pH 6.5) was measured by amplifying 61bp, 94 bp and 127 bp fragments in the *BCR* gene and 61 bp and 98 bp fragments in the *N-ras* gene. Lane 2: no preheating, lane 8: preheated with TE (pH 7.0) for 30 min, lane 9: negative control, lane 10: positive control, lane 1: pUC19-*Msp*I digest for DNA size marker. The bands different from the position shown by arrow indicate the extra bands. This is the same in Figures 2 - 5. The bar graphs at right indicate the relative intensity of each target band when intensity of positive control is assumed to be 1.0. The numbers on the horizontal axis correspond to the number of lanes in the left electrophoresis. This labeling is also used in Figures 3 - 5.

Figure 4. Detailed analysis of pH of citrate buffer ranging from 3.0 to 5.0 for optimization of preheating condition. RNA was preheated in citrate buffer (lane 3: pH 3.0, lane 4: pH 3.5, lane 5: pH 3.7, lane 6: pH 4.0, lane 7: pH 4.25, lane 8: pH 4.5, lane 9: pH 5.0) at 70C for 30min before cDNA was synthesized. RT-PCR amplification of *BCR* and *N-ras* mRNAs was carried out to determine the optimal pH for preheating of RNA. Lane 2: no preheating, lane 10: preheated with TE (pH 7.0) for 30 min, lane11: negative control, lane 12: positive control, lane 1: pUC19-*Msp*I digest for DNA size marker.

Figure 5. Effect of concentration of citrate buffer on RT-PCR amplification of RNA isolated from archival thyroid tissues. RT-PCR amplification of RNA preheated at 70C for 30 min in various concentrations of citrate buffer with pH 4.0 (lane 3: 50 mM, lane 4: 10 mM, lane 5: 2 mM) was performed on three different sizes of fragments in the *BCR* gene and two different sizes of fragments in the *N-ras* gene. Lane 2: no preheating, lane 6: negative control, lane 7: positive control, lane 1: pUC19-*Msp*I digest for DNA size marker.

Figure 6. Effect of preheating time on RT-PCR amplification. RT-PCR amplification of RNA preheated at 70C in citrate buffer (pH 4.0) for various preheating times (lane 2: 0 min, lane 3: 10 min, lane 4: 20 min, lane 5: 30 min, lane 6: 45 min, lane 7: 60 min, lane 8: 90 min, lane 9: 120 min) was done for different lengths of fragments in *BCR* and *N-ras*. Lanes 10 and 11: preheated with TE for 30 min and 60 min, lane 12: negative control, lane 13: positive control, lane 1: pUC19-*Msp*I digest.

Figure 7. Application of preheating in citrate buffer (pH 4.0) to RT-PCR amplification of RNA extracted from the other four archival thyroid tissues (A – D). RNA was heated in citrate buffer at 70C for 45 min before cDNA was synthesized (lane 3). Lane 2: no preheating, lane 4: negative control, lane 5: positive control, lane 1: pUC19-*Msp*I digest.

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Figure 1

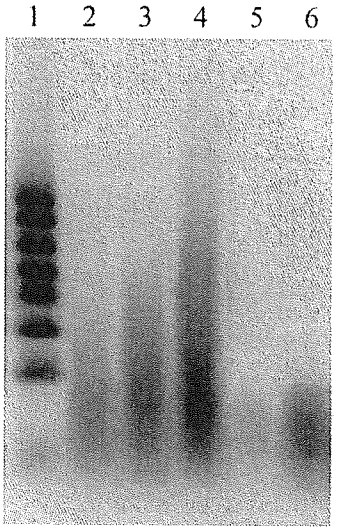

Figure 2

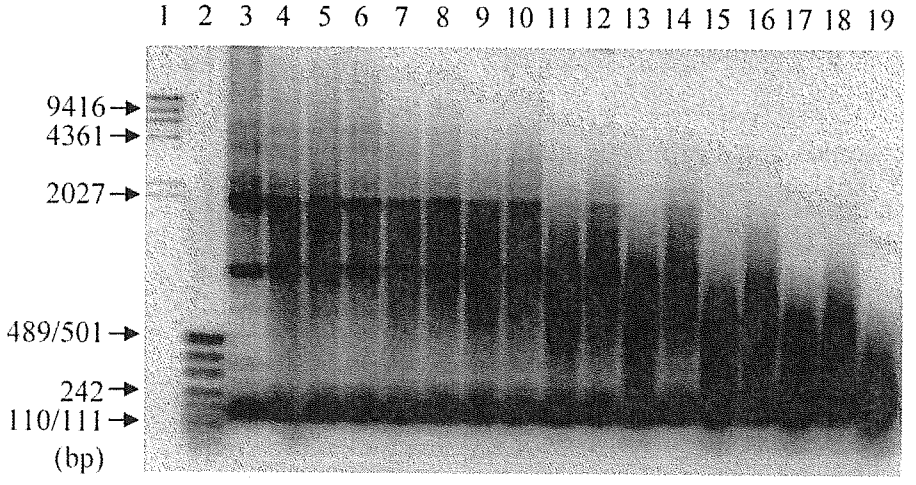

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Figure 3

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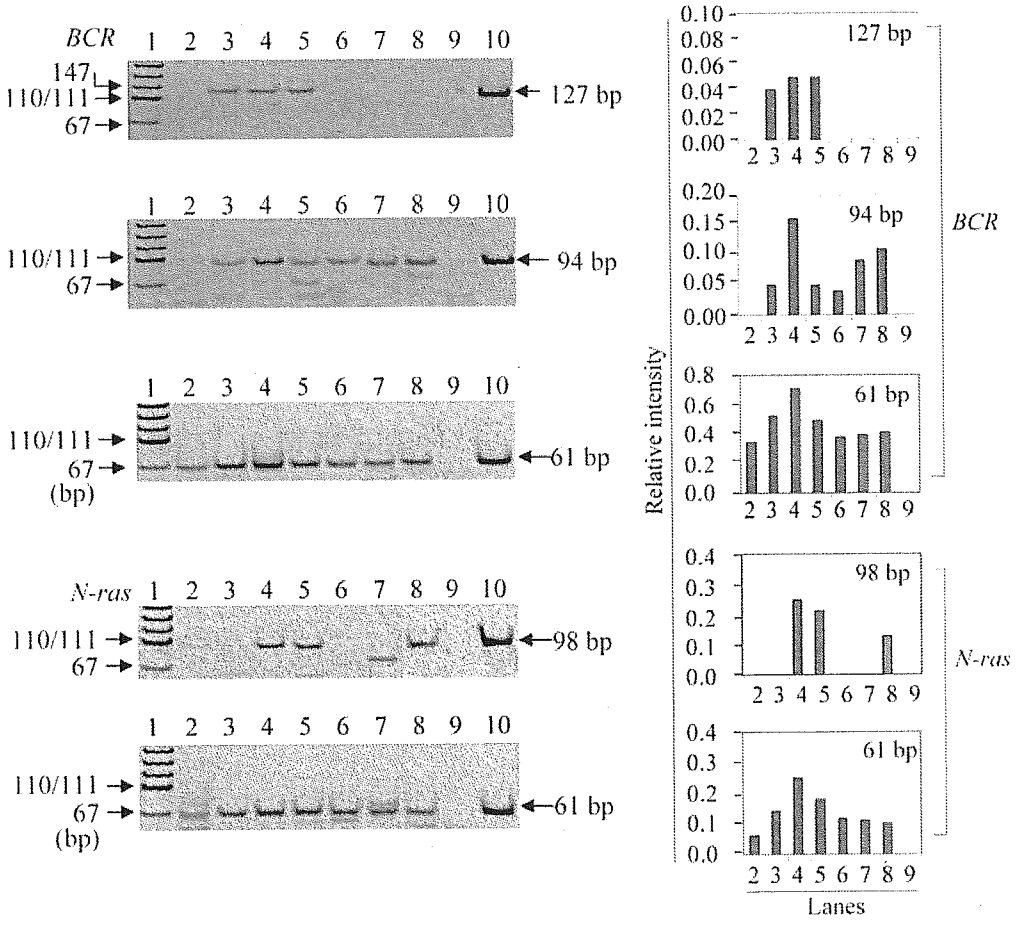

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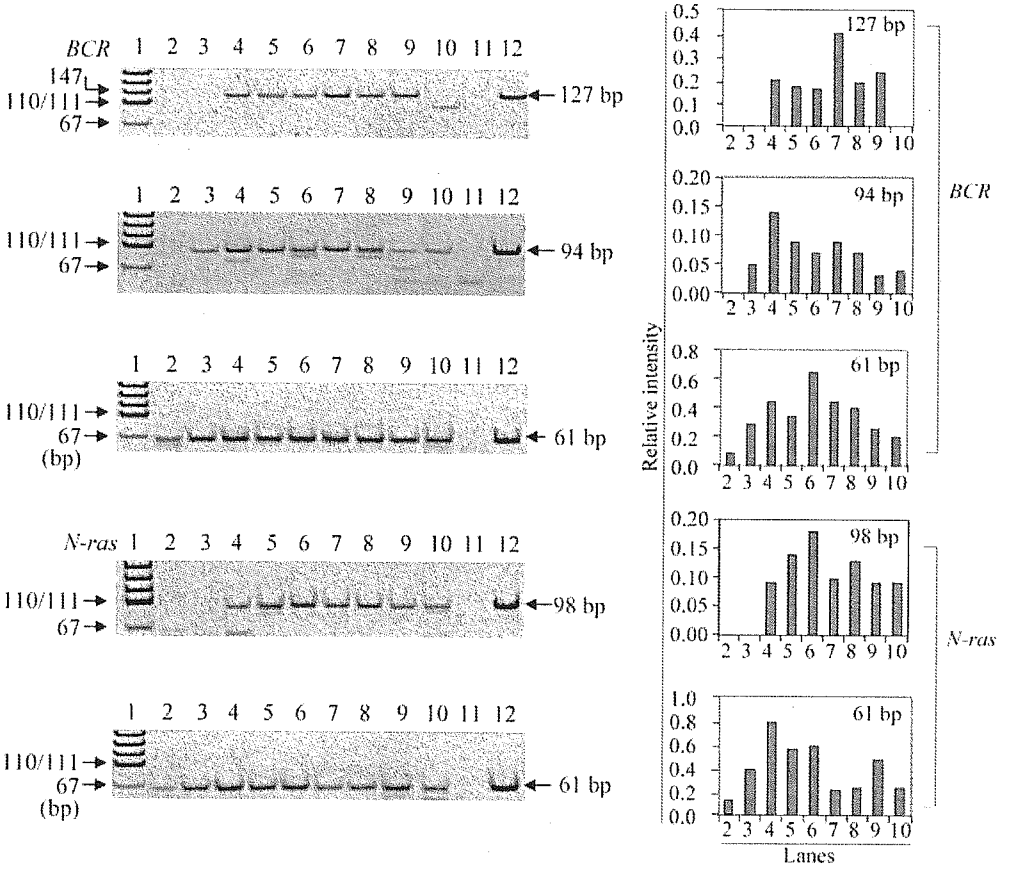

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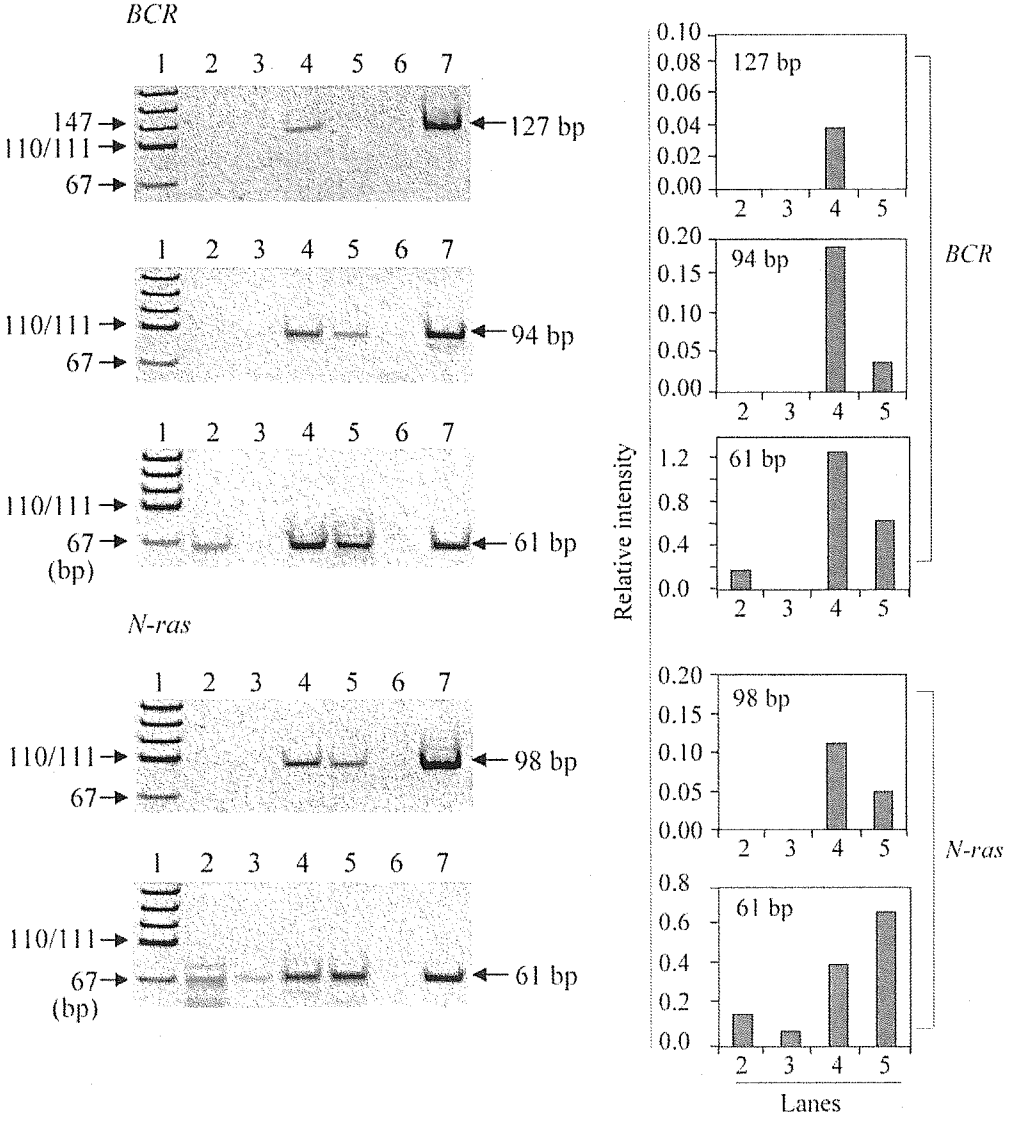

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Figure 6

Figure 6

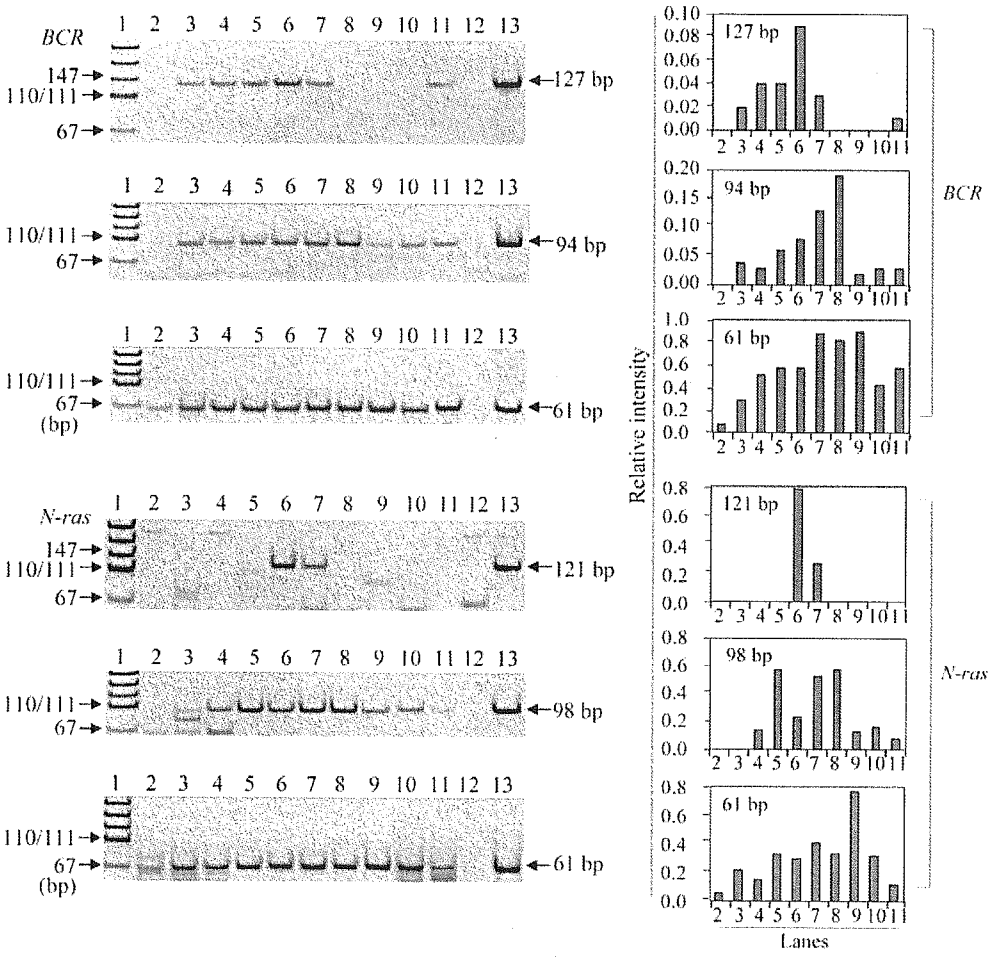

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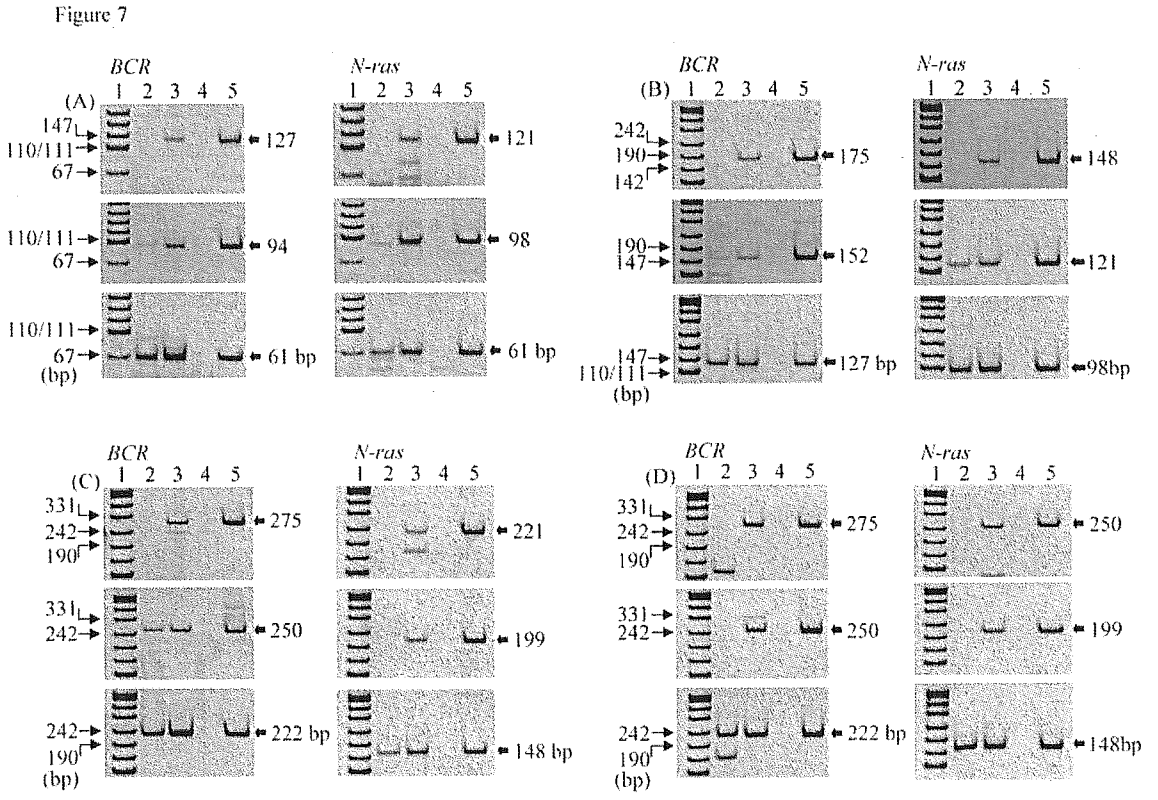
Figure 7



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Long-term effects of radiation dose on inflammatory markers in atomic bomb survivors

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BRIEF OBSERVATION

Long-term effects of radiation dose on inflammatory markers in atomic bomb survivors

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Atomic bomb survivors have a persistently increased risk of cancer, hepatitis, and cardiovascular and autoimmune diseases.[1-4] There is no clear explanation for these late effects of radiation exposure. One hypothesis is that radiation causes chronic low-grade inflammation, with elevated circulating levels of cytokines. Proinflammatory cytokines, such as interleukin 6, tumor necrosis factor (TNF) α, and interferon γ, and anti-inflammatory cytokines, such as interleukin 10, are synthesized predominantly by macrophages and lymphocytes, and regulate the inflammatory response.[5,6] Interleukin 6 in turn induces the synthesis of acute-phase plasma proteins, such as C-reactive protein.[7] Increased levels of inflammatory cytokines, even within the normal range, have been associated with an increased risk of cardiovascular disease.[8] Chronic low-grade inflammation may also influence the production of immunoglobulins by B cells.[9]

We therefore analyzed the effects of presumed radiation dose on inflammatory parameters in atomic bomb survivors.

Methods

Subjects

We studied subjects from Hiroshima who had participated in an epidemiological follow-up study of atomic bomb survivors, which collected health information from 2436 survivors during biennial medical examinations.[10] Peripheral blood samples were collected between March 1995 and April 1997. We obtained institutional approval from the human investigation committee and informed consent from participants. We excluded subjects with a history of cancer or diseases that have been associated with inflammation (e.g., current upper respiratory tract infection, chronic bronchitis, collagen disease, arthritis, or myocardial infarction). We classified the other participants into four radiation dose groups: nonexposed, low dose (0.005 to 0.7 Gy), medium dose (0.7 to 1.5 Gy), and high dose (>1.5 Gy). Estimated bone marrow doses were based on the 1986 Dosimetry System.[11] Doses were for whole-body exposure, mainly from gamma rays but with a small neutron component. We selected 180 subjects from the nonexposed group and 90 from each of the other

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Table 1 Characteristics of the study subjects*

Characteristic	Nonexposed (n = 179)	Radiation Exposure (Gy) 0.005–0.7 (n = 87)	0.7–1.5 (n = 88)	>1.5 (n = 88)
	Number (%) or mean ± SD			
Radiation dose (Gy)	0	0.3 ± 0.2	1.1 ± 0.2	2.1 ± 0.5
Age (years)	68 ± 11	69 ± 11	67 ± 10	68 ± 10
Female sex	96 (54)	50 (58)	52 (59)	47 (53)
Body mass index (kg/m^2)	23 ± 3	23 ± 3	22 ± 4	23 ± 4
Current smokers	44 (25)	17 (20)	23 (26)	21 (24)

*Among atomic bomb survivors from Hiroshima, Japan.

groups, such that the age and sex distributions were similar in the four groups. Data were missing for 8 subjects; these subjects were excluded from all analyses.

Measurements

We measured plasma TNF-α, interferon γ, and interleukin 10 levels in duplicate using a highly sensitive enzyme-linked immunosorbent assay kit (Quantikine HS; R&D Systems, Minneapolis, Minnesota). Mean values of duplicate measurements were reported for all assays. We quantitated immunoglobulin levels using standard kits (Bethyl Lab. Inc., Montgomery, Texas). The interassay and intra-assay coefficients of variations of these enzyme-linked immunosorbent assay kits were lower than 10%. The erythrocyte sedimentation rate was measured using standard methods.

Statistical analysis

We estimated the effects of changes in several predictor variables (linear radiation dose, age, and sex), adjusted for current smoking and body mass index (in kg/m^2) using a multivariate linear regression model based on the log of the outcome variables (biological markers). We present results as percentage changes in the outcome variables with 95% confidence intervals. All analyses were performed using SAS software (Cary, North Carolina).

Results

There were no significant differences in age, sex, body mass index, or current smoking among the four groups (Table 1). Interferon γ levels and the erythrocyte sedimentation rate increased significantly with radiation dose (Figure). Tumor necrosis factor α and interleukin 10 levels also increased slightly but not significantly with radiation dose. The levels of immunoglobulin (Ig) A and IgM increased significantly with radiation dose, but those of IgG and IgE did not.

In multivariate models, the levels of TNF-α, interferon γ, and interleukin 10, and the erythrocyte sedimentation rate, increased significantly with radiation dose, as did IgA, IgM, and total immunoglobulin levels (Table 2). The levels of TNF-α, interleukin 10, IgG, IgA, and total immunoglobulins, and the erythrocyte sedimentation rate, increased significantly with age.

Discussion

Tumor necrosis factor α, interleukin 6, interferon γ, and interleukin 10 coordinate the inflammatory response. In the present study, plasma levels of inflammatory cytokines and biomarkers (TNF-α and the erythrocyte sedimentation rate) increased with radiation dose and with age. Plasma levels of other cytokines (interferon γ and interleukin 10) and immunoglobulins (IgA and IgM) increased with radiation dose. Combined with previous results on other inflammatory signs, such as increased white blood cell counts, and sialic acid and C-reactive protein levels,[12,13] our results provide evidence of persistent inflammatory responses in atomic bomb survivors more than 50 years after radiation exposure.

In light of these studies, we hypothesized that radiation exposure accelerated aging. To test the hypothesis, we calculated radiation exposure as a function of age using inflammatory status as an index. We estimated that exposure to 1 Gy was equivalent to an increase in age of about 9.0 years. Noting that the mean exposure among atomic bomb survivors was about 0.2 Gy, we inferred that mean accelerated aging among atomic bomb survivors was about 2 years (range, 1 to 2.5 years). Others have reported that the decrease of CD4-expressing T cells was about 4% per 10 years and 2% per Gy, implying that the decrease per Gy is equivalent to about 5 years of aging.[14] Furthermore, interleukin 6 levels correlate

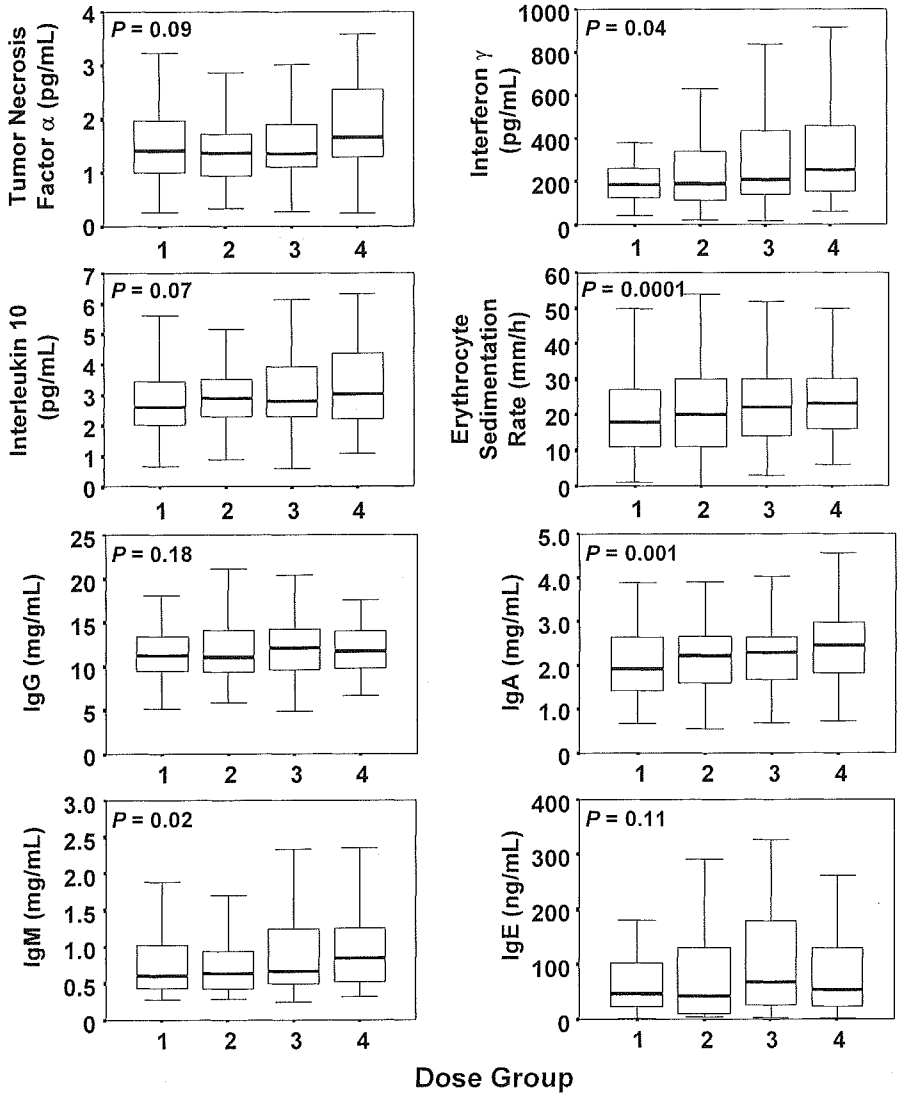

Figure 1 Box plot of inflammatory biomarker levels and erythrocyte sedimentation rate among atomic bomb survivors. The horizontal line inside the box represents the median. Lower and upper boundaries represent the 25th and 75th percentiles. Whiskers represent the smallest and largest values that are less than 1.5 box-length from the 25th and 75th percentiles. 1 indicates nonexposed; 2 indicates radiation exposure of 0.005 to 0.7 Gy; 3 indicates exposure of 0.7 to 1.5 Gy; and 4 indicates exposure of >1.5 Gy. Ig = immunoglobulin.

negatively with the percentage of CD4 T cells.[13] Thus, acceleration of immunological aging may also be involved in radiation effects on the inflammatory status in humans.

Increased mortality and morbidity from cardiovascular disease has been observed in atomic bomb survivors,[10] and elevated plasma levels of inflammatory markers, including interleukin 6, have been associated with an increased risk of cardiovascular disease.[8] Indeed, C-reactive protein and complement are mediators of ischemic myocardial injury.[15] Further, the percentage of CD4 T cells in the blood is markedly lower among atomic bomb survivors who have a history of myocardial infarction.[16] We hypothesize that modification of cytokine production may be involved in the onset or progression of some of the conditions, such as hepatitis and cardiovascular disease, which are more common in atomic bomb survivors.

Several studies have shown that radiation causes short-term inflammatory effects, such as increased plasma levels of proinflammatory cytokines, among patients who received radiation therapy.[17-19] In addition, radiation for cancer or Hodgkin's disease leads to long-term depletion of naïve CD4 T cells,[20,21] and pathologic cardiac changes.[22] Our results suggest that radiation exposure may also produce long-term adverse effects by generating a persistent inflammatory status, manifested by cytokines and other inflammatory markers along with long-lasting impairment of CD4 T cells. Given the potential implication of our findings, follow-up of radiotherapy-treated patients is warranted to assess the asso-

Table 2 Multivariate models of the effects of age, sex, and radiation dose on inflammatory biomarkers and immunoglobulins

Variable	Tumor Necrosis Factor α	Interferon γ	Interleukin 10	Erythrocyte Sedimentation Rate	Total Ig	IgG	IgA	IgM	IgE
	Percentage Increment (95% Confidence Interval)								
Age per 10 years	15 (9 to 20)	4 (−4 to 12)	8 (4 to 13)	15 (9 to 20)	3 (1 to 6)	3 (1 to 6)	5 (2 to 9)	−6 (−11 to 14)	2 (−11 to 14)
Female sex*	15 (2 to 30)	−8 (−23 to 10)	6 (0 to 12)	17 (9 to 24)	5 (0 to 10)	7 (1 to 13)	−9 (−17 to −1)	14 (1 to 28)	−51 (−63 to −34)
Radiation dose per Gy	7 (1 to 15)	12 (2 to 23)	6 (0 to 12)	17 (9 to 24)	3 (1 to 6)	2 (−1 to 5)	8 (3 to 13)	9 (2 to 15)	14 (−3 to 32)

Ig = immunoglobulin.
*Compared with men.

ciation between inflammatory status and the occurrence of inflammation-associated diseases.

References

1. Pierce DA, Shimizu Y, Preston DL, et al: Studies of the mortality of atomic bomb survivors. Report 12, Part I. Cancer: 1950-1990. Radiat Res 146:1–27, 1996
2. Kodama K, Sasaki H, Shimizu Y: Trend of coronary heart disease and its relationship to risk factors in a Japanese population: a 26-year follow-up, Hiroshima/Nagasaki study. Jpn Circ J 54:414–421, 1990
3. Sharp GB, Mizuno T, Cologne JB, et al: Hepatocellular carcinoma among atomic bomb survivors: significant interaction of radiation with hepatitis C virus infections. Int J Cancer 103:531–537, 2003
4. Nagataki S, Shibata Y, Inoue S, et al: Thyroid diseases among atomic bomb survivors in Nagasaki. JAMA 272:364–370, 1994
5. Van der Meide PH, Schellekens H: Cytokines and the immune response. Biotherapy 8:243–249, 1996
6. Tracey KJ: The inflammatory reflex. Nature 420:853–859, 2002
7. Fey GH, Hattori M, Hocke G, et al: Gene regulation by interleukin 6. Biochimie 73:47–50, 1991
8. Ridker PM, Rifai N, Stampfer MJ, et al: Plasma concentration of interleukin 6 and the risk of future myocardial infarction among apparently healthy men. Circulation 101:1767–1772, 2000
9. Papadaki HA, Eliopoulos DG, Ponticoglou C, et al: Increased frequency of monoclonal gammopathy of undetermined significance in patients with nonimmune chronic idiopathic neutropenia syndrome. Int J Hematol 73:339–345, 2001
10. Kodama K, Mabuchi K, Shigematsu I: A long-term cohort study of the atomic-bomb survivors. J Epidemiol 6:S95–105, 1996 (suppl)
11. Roesch WC: US-Japan Joint Reassessment of Atomic Bomb Radiation Dosimetry in Hiroshima and Nagasaki Hiroshima, Japan: Radiation Effects Research Foundation; 1987
12. Neriishi K, Nakashima E, Delongchamp RR: Persistent subclinical inflammation among A-bomb survivors. Int J Radiat Biol 77:475–482, 2001
13. Hayashi T, Kusunoki Y, Hakoda M, et al: Radiation dose-dependent increases in inflammatory response markers in A-bomb survivors. Int J Radiat Biol 79:129–136, 2003
14. Kusunoki Y, Kyoizumi S, Hirai Y, et al: Flow cytometry measurements of subsets of T, B and NK cells in peripheral blood lymphocytes of atomic bomb survivors. Radiat Res 150:227–236, 1998
15. Griselli M, Herbert J, Hutchinson WL, et al: C-reactive protein and complement are important mediators of tissue damage in acute myocardial infarction. J Exp Med 190:1733–1740, 1999
16. Kusunoki Y, Kyoizumi S, Yamaoka M, et al: Decreased proportion of CD4 T cells in the blood of atomic bomb survivors with myocardial infarction [published erratum appears in Radiat Res 154:119, 2000]. Radiat Res 152:539–543, 1999
17. Wickremesekera JK, Chen W, Cannan RJ, et al: Serum proinflammatory cytokine response in patients with advanced liver tumors following selective internal radiation therapy (SIRT) with (90)Yttrium microspheres. Int J Radiat Oncol Biol Phys 49:1015–1021, 2001
18. Wichmann MW, Meyer G, Adam M, et al: Detrimental immunologic effects of preoperative chemoradiotherapy in advanced rectal cancer. Dis Colon Rectum 46:875–887, 2003
19. Barthelemy-Brichant N, Bosquee L, Cataldo D, et al: Increased IL-6 and TGF-beta1 concentrations in bronchoalveolar lavage fluid associated with thoracic radiotherapy. Int J Radiat Oncol Biol Phys 58:758–767, 2004
20. Mackall CL, Hakim FT, Gress RE: T-cell regeneration: all repertoires are not created equal. Immunol Today 18:245–251, 1997
21. Watanabe N, De Rosa SC, Cmelak A, et al: Long-term depletion of naive T cells in patients treated for Hodgkin's disease. Blood 90:3662–3672, 1997
22. Friedlander AH, Sung EC, Child JS: Radiation-induced heart disease after Hodgkin's disease and breast cancer treatment: dental implications. J Am Dent Assoc 134:1615–1620, 2003