Evaluation of DNA Double Strand Breaks in Human and Mouse Lymphocyte Following γ-Irradiation

Tae Hwan Kim, D.V.M. Ph.D., Sung Ho Kim, D.V.M. Ph.D., In Yong Chung M.S., Chul Koo Cho, M.D.,* Kyung Hwan Koh, M.D.* and Seong Yul Yoo, M.D.*

Laboratory of Radiation Medicine and Department of Therapeutic Radiology, KCCH, KAERI*

The evaluation of radiation-induced DNA double strand breaks (DSB) was made following irradiation of human lymphocytes, murine lymphocytes and EL-4 leukemia cells over a wide dose range of ^{60}Co γ-rays. In lipopolysaccharide (LPS) or phytohemagglutinin (PHA)-stimulated murine lymphocytes, the slopes of the strand scission factor (SSF) revealed that lymphocytes with LPS increased DNA DSB formation by a factor of 1.432 ($p<0.005$). Furthermore, strand break production was relatively inefficient in the T lymphocytes compared to the B lymphocytes. And EL-4 leukemia cells were found to form significantly more DNA DSB to a greater extent than normal lymphocytes ($p<0.005$).

The in vitro studies of the intrinsic radiosensitivity between human lymphocytes and murine lymphocytes showed similar phasic kinetics. However, murine lymphocytes were lower in DNA DSB formation and higher in the relative radiation dose of 10 percent DNA strand breaks at 3.5 hours following γ-irradiation than human lymphocytes. Though it is difficult to interpret these results, these differences may be result from environmental and genetic factors.

From our data, if complementary explanations for this difference will be proposed, the differences in the dose-effect relationship for the induction of DSB between humans and mice must be related to interspecies variations in the physiological condition of the peripheral blood in vitro and not to differences in the intrinsic radiation sensitivity of the lymphocytes. These results can be estimated on the basis of dose-effect correlation enabling the interpretation of clinical response and the radiobiological parameters of cytometrical assessment.

Key Words: γ-irradiation, DNA double strand breaks, Lymphocytes, Neutral elution

INTRODUCTION

Current radiobiological research is evaluating the relationships between radiation induced DNA lesions at the molecular level and radiobiological phenomena observed at the cellular or tissue level. Ionizing radiation induces a range of lesions in cellular DNA. The role of the various DNA lesions induced by radiation in cell killing has been the subject of much debate. There is now considerable evidence that there is a poor correlation between the initial level of these lesions and cell death. Also DNA double strand breaks have attracted much attention as candidates for the role of lethal lesions in radiation-induced cell lethality[1~7]. There is evidence showing that chromosome aberrations can be produced by double strand breaks[8~10]. While cell death after exposure to ionizing radiation is thought to be predominantly a consequence of damage to the DNA. A measure of some component of this damage can be made by assessing the number of radiation-induced DNA single strand breaks using the alkaline elution method[11]. Studies in mammalian cells have proposed that unrepaired double strand breaks were responsible for cell lethality, and models relating these to the formation of radiation-induced cellular chromosome changes have been formulated[12~14].

Until recently the overall incidence of double strand breaks as measured by neutral filter elution[15] correlated with survival has allowed the measurement of double strand breaks in the same dose range as cell survival experiments, avoiding any of the uncertainties of the extrapolations that were required in earlier work. Using the filter elution technique a curvilinear relationship between radiation induced double strand breaks and dose was nearly obtained which mirrors a cells survival curve[16]. Despite the wide use of the filter elution technique, systematic studies on the parameters that determine DNA elution are lacking, although important conclusions regarding the mechanism of the action of radiation on living cells have been

based on the shape of the DNA elution dose response curves.

For these reasons, a series of experiments were initiated to study DNA elution dose-response in murine lymphocytes, EL-4 leukemia cells and human lymphocytes using the filter elution technique in this paper, and we have now compared human lymphocytes irradiated in vitro to murine lymphocytes in order to evaluate the level of induced damage in the determination of the exposed radiation dose.

MATERIALS AND METHODS

1. Animals and Subjects

Female C57BL/6 mice, aged 8 weeks, were obtained from our division of experimental animal care and management. The animals were kept in polycarbonate cages and were allowed NIH-07 pelleted food and water ad libitum. Animal quarters were conditioned at 23℃, 60 per cent relative humidity, and 12 hour light and dark cycles. And healthy laboratory staff provided blood for control experiments. Venous blood was collected from the cubital vein into sterile heparinized vacutainer tubes by venipuncture.

2. Cell Line

The EL 4 leukemia cell line was derived from C57BL/6 mice. Cultures were maintained in tissue culture flasks at 37℃ in humidified 95 air 5 carbon dioxide environment. The cell line has a population doubling time of approximately 8 hours.

3. Lymphocytes

Human fresh blood was mixed with phosphate-buffered saline (PBS) in the proportions 1:1 and was layered on the top of a mixture of Ficoll-hypaque solution. Extracted mice spleens were washed with Hank's balanced salt solution (HBSS). For the separation of the lymphocytes, they were minced in a petri dish containing 10 ml of HBSS with the resultant cell suspension. The cell suspension was added to the Ficoll-hypaque solution. After centrifugation at 400 g for 30~40 minutes at 20℃, the lymphocytes were separated from other blood components with a Pasteur pipette and washed in cold PBS solution. The lymphocytes having 5×10^5 cells/ml with less than 3 percent erythrocytes were seeded in tissue culture flasks with RPMI medium 1640 containing 5 per cent fetal calf serum, 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 25 μg/ml of LPS or 2.5 μg/ml of PHA maintained at 37℃ in a 5 percent CO_2 atmosphere. 2 μCi/ml of [^{3}H]-thymidine added to each culture 4 hours before harvesting. The maximum uptake of [^{3}H]-thymidine in murine lymphocytes and human lymphocytes occurred about 48 hours and 72 hours respectively after stimulation with PHA. After this labelling period, the cells were pulsed-chased in [^{3}H]-thymidine free medium for a period of 2 hours to reduce the amount of labelled low molecular weight DNA.

4. Irradiation

In this experiment, 1×10^6 cells/ml in the medium were placed in sterile 24 well bottom culture plate (Flow lab. Inc.) and kept on ice until they were irradiated with a ^{60}Co γ-ray from a theratron-780 teletherapy unit (Picker, V4M60) at a dose rate of 1.3 Gy min^{-1} and exposed to graded single doses of 25, 50, 75 and 100 Gy. Immediately after irradiation, the suspension was diluted with ice-cold PBS to ensure inhibition of DNA repair[17].

5. Filter Elution

The filter elution technique used was essentially that described by Bradley and Kohn[15], with a slight modification that was used to determine DNA damage induction by measuring the rate of elution of double stranded DNA through a filter at neutral pH 9.6. Prior to assay, cultures were grown in the presence of [^{3}H]-thymidine 48 hours for murine lymphocytes, 16 hours for EL-4 leukemia cells and 72 hours for human lymphocytes, respectively. 1×10^6 cells/ml were suspended in RPMI 1640 medium and then cells were washed and collected in ice-cold PBS on to 25 mm diameter polycarbonate filters (1.2 μm pore size, Nucleopore Corporation). The cells were then lysed for 30 minutes at room temperature in 0.05M Tris, 0.05M glycine, 0.025M Na_2EDTA, 2 percent (w/v) sodium lauryl sulphate and, just prior to use, 0.5 mg/ml proteinase K (Sigma) was added. After 30 minutes of lysis, the solution was allowed to drip through the filters by gravity. An elution buffer consisting of lysing solution besides proteinase K was added in the dark and pumped through the filter at a rate of 0.1 ml/min. Fractions were collected every 30 minutes for up to 5 hours.

6. Strand Scission Factor Calculation (SSF)

The designation of SSF refers to a relative value determined by comparison of associated DNA elution curves. This value is used to characterize

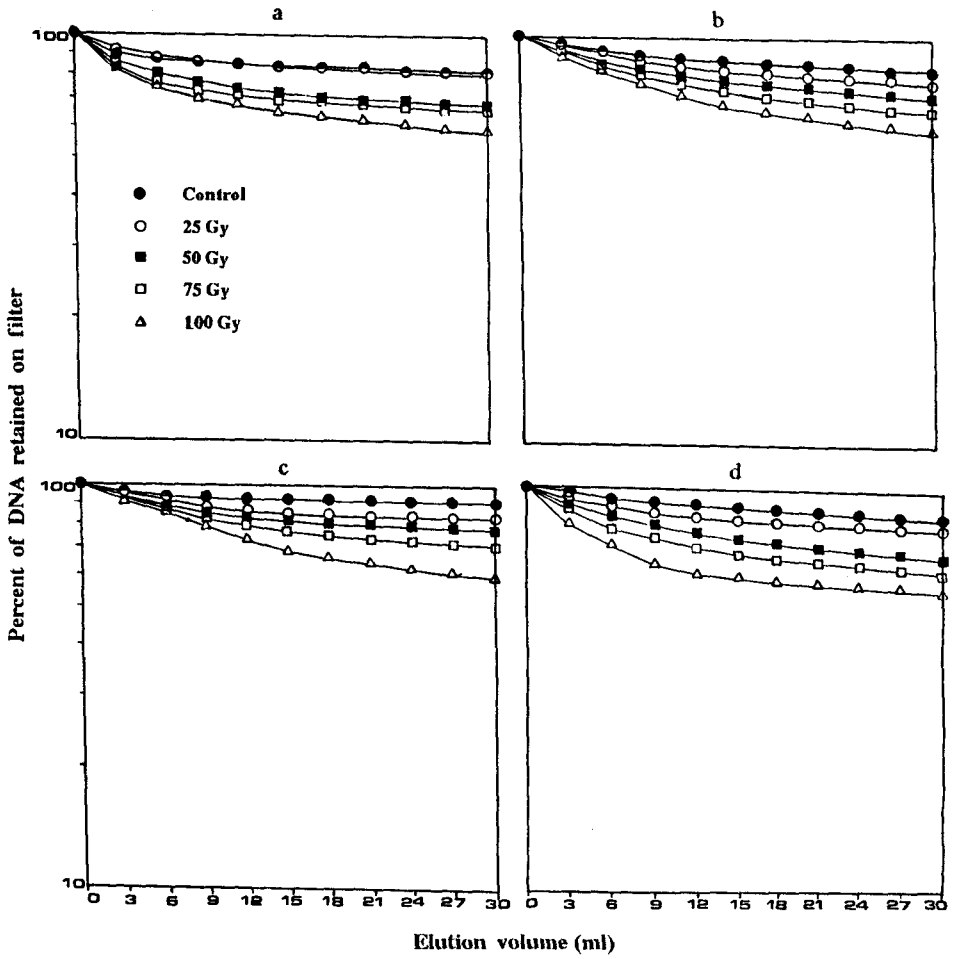

Fig. 1. Percent of PHA (a, b) or LPS (d) stimulated lymphocytes DNA retained on the filter versus elution volume in DNA double strand break assay. The cells in suspension were exposed at 0℃ to each dose of ^{60}Co radiation. Figures are shown human lymphocytes (a), mouse lymphocytes (b, d), and EL4 leukemia cells (c).

relative numbers of DNA strand breaks. Specifically, SSF was determined from: $SSF=[\log''\{(f_x)/(f_o)\}]$, where f_o and f_x are the proportions of DNA retained on the filter after an eluted volume of 30 ml for the unirradiated and irradiated samples respectively[17]. Calculation of the relative elution at this elution volume has recently been shown to correlate with double strand breaks[18].

7. Statistical Analysis

The points shown in Fig. 1, 2 are means of triplicate determinations±SD. The computer fitting of lines figures was plotted as mean±SD. Statistical analysis of SSF values was performed using the student T-test.

RESULTS

1. Evaluation of DNA Double Strand Breaks

For the evaluation of DNA DSB formation on murine lymphocytes, EL-4 leukemia cells and human lymphocytes, typical neutral elution profiles for DNA from cell lines after irradiation with various doses of ionizing radiation are shown in Fig. 1. This figure shows a semi-log plot of the fraction of [^{3}H]-labelled DNA retained on the filter versus time of elution procedures. At each dose tested, DSB formation was increased by increasing the dose of irradiation. The same experimental design with a

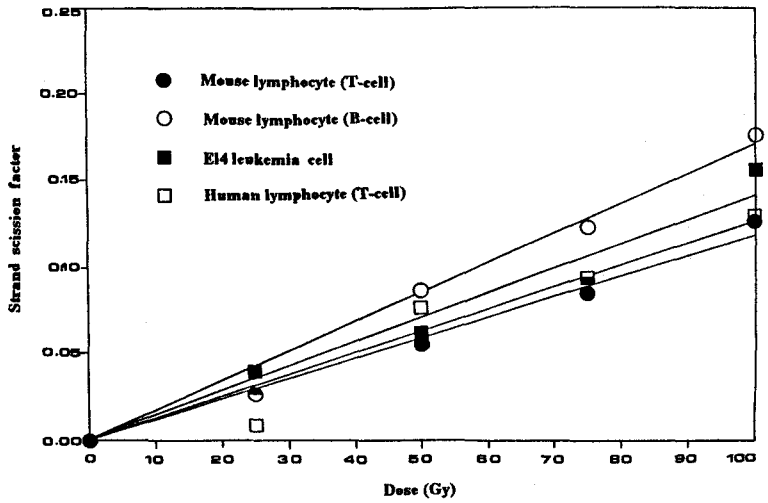

Fig. 2. DNA dsb in the cells measured by neutral elution at 3.5 hours following gamma irradiation.

Table 1. Comparision of the Relative Number of K_d Per Radiation Dose

Experimental group	Cell	Mitogen	K_d (Gy^{-1})*($n=5$)
I	Human lymphocyte	PHA	0.001264±0.0000992
II	Mouse lymphocyte	PHA	0.001192±0.0000354
III	Mouse lymphocyte	LPS	0.001707±0.0000571
IV	EL-4 leukemia cell	–	0.00142 ±0.0000765

*K_d; Slope of strand scission factor (SSF) of DNA double strand breaks.

cultivation condition after PHA caused more a reduction in the detection of DSB formation for all doses tested than after LPS. The linear dose response relationship for SSF indicates a quality of DNA damage which is directly proportional to radiation dose on all of the tested cells (Fig. 2). The murine lymphocytes with LPS had a slope of 0.001707±0.0000571 Gy^{-1} (n=5), while lymphocyte proliferation with PHA had a slope of 0.001192±0.0000354 Gy^{-1} (n=5). The ratio of the two slopes in the degree of radiation sensitivity reveals that murine lymphocytes with PHA reduced DSB formation by a factor of 1.432 ($p<0.005$).

2. Comparison of the Radiosensitivity Between PHA-Stimulated Human Lymphocytes and Murine Lymphocytes

In a comparison of PHA-stimulated lymphocytes and murine lymphocytes, the DSB formation of these lymphocytes was increased in proportion to the radiation dose. The SSF values for human lymphocytes had a slope of 0.001264±0.0000992 Gy^{-1} (n=5). The in vitro studies of the intrinsic radiosensitivity between human lymphocytes and murine lymphocytes showed similar phasic kinetics. However, murine lymphocytes were lower in DNA DSB formation and higher in the relative radiation dose of 10 percent DNA strand breaks at 3.5 hours following γ-irradiation than human lymphocytes (Fig. 1, 2).

Table 1 and 2 show an analogous tendency to the above results in the sensitivity differences between normal and tumor tissue in mice to ionizing radiation-induced strand breaks in vitro. PHA-stimulated normal lymphocytes had a slope of 0.001192±0.0000354 Gy^{-1} (n=5), while EL-4 leukemia cells had a slope of 0.00142±0.0000765 Gy^{-1} (n=5). The ratio of the two slopes in the degree of radiation sensitivity reveals that EL-4 leukemia cells increased DSB formation by a factor of 1.191 ($p<0.005$).

DISCUSSION

Much of the interest in studying the biological effect of ionizing radiation on cells has been based

Table 2. Comparison of the Relative Radiation Dose of 10 Per Cent DNA Strand Breaks at 3.5 Hours Following γ-irradiation

Elution type	Cell	Dose (Gy)
Neutral elution	human lymphocyte	78.74
	mouse lymphocyte (PHA)	84.55
	mouse lymphocyte (LPS)	59.09
	EL4 leukemia cell	71.40

on DNA DSB formation. Radiation induced-DNA double strand breaks are believed to be important lesions that are related to cell killing, induction of chromosome aberrations and carcinogenesis. That DNA double strand breaks may be the critical lesions leading to cell death in mammalian cells is suggested by the observation that induction of DNA double strand breaks by restriction endonucleases leads to the induction of chromosome aberrations and cell death[1,9,10,15].

In order to establish a quantitative correlation between induction of DNA double strand breaks in mammalian cells and radiation dosage, accurate measurement of their induction per unit absorbed dose is required. Methods presently used to measure the induction of DNA double strand breaks include velocity sedimentation, filter elution, gel electrophoresis and viscoelastometry. However, due to the high molecular weight of the mammalian cell DNA, accurate measurement for the induction of double strand breaks has been difficult, and conflicting results were occasionally obtained by investigators using different techniques[18~20].

Therefore, the present investigation carried out accurate measurement of DNA DSB formation using filter elution under conditions that prevent denaturation of the DNA molecules, and that measured the quantitative correlation between induction of DNA double strand breaks and radiation dose in normal murine lymphocytes, human lymphocytes and EL-4 mouse leukemia cells. A major advantage of this technique, apart from its ease of use, is that it allows measurement at radiation doses of the exposed person within the dose-response range thus enabling correlations to be established between absorbed dose and DNA double strand breaks induction. In this experiment, these findings show that a direct correlation of the DNA filter elution behaviour with the induction of DNA, may not always be justified, and they establish correlations between the level of induction of DNA and dose. The induction of DNA DSB in PHA-stimulated lymphocytes is shown to be closely correlated with dose, but formed lower DSB formation than LPS-stimulated lymphocytes. Our present data are substantially in accordance with previous data[22] that B lymphocytes have more radiosensitivity than T lymphocytes.

And the radiosensitive effect between normal murine lymphocytes and EL-4 leukemia cells at the rate of double strand breaks formation was not surprising, but EL-4 leukemia cells were found to form significantly more DNA DSB, and to greater extent than normal lymphocytes. An elevated efficiency of radiation induced DNA DSB production in undifferentiated EL-4 leukemia cells may be due to the proliferative activity of the cells. Our data can partially explain that the radiosensitivity of various tissues, when determined by measurements of DNA DSB formation, varies remarkably from tissue to tissue in the animal and when compared to irradiation of the cells in vitro.

Also there is little information concerning the radiation sensitivity of human lymphocytes isolated directly from normal persons, even though the radiation response of these is often dose limiting for whole body and partial irradiation. This report describes the radiation dose-response data for human lymphocytes and murine lymphocytes. Complete dose response curves were generated for human peripheral blood cells irradiated in vitro under conditions that would mimic those of the murine lymphocytes. They were irradiated in tissue culture medium at 0℃. In a comparison of irradiation at 37℃ to that on ice, that the slopes of the dose response curves that were identical to each other was reported by Meyn and Jenkins[17]. The in vitro studies of the intrinsic radiosensitivity between human lymphocytes and murine lymphocytes nearly all assume a constant proportion of DSB formation after each dose fraction, but murine lymphocytes were lower in DNA DSB formation and higher in the relative radiation doses of 10 percent DNA strand breaks at 3.5 hours following γ-irradiation than human lymphocytes. The results reported here may result from environmental and genetic factors. While we can only speculate as to the meaning of these results, it seems possible that this interspecies variation in DNA strand breaks formation between human lymphocytes and murine lymphocytes may be due to the genetic differences that occur among tissues in living animals.

These findings can be used to establish correla-

tions between the level of induction of DNA DSB and the radiation dose in interpretation of clinical response and the radiobiological parameters of cytometrical assessment.

ACKNOWLEDGEMENTS

The authors would like to think Kim, Kyeong-Jung for technical help with cell culture and irradiation, Woo, Kang-Sun for statistical analysis and Chung, Young-Sook for typing the manuscript.

REFERENCES

1. Wolff S, AFzal V, Wiencke JK, Olivieri G, Michaell A: Human lymphocytes exposed to low doses of ionizing radiations become refractory to high doses of radiation as well as to chemical mutagens that induce double-strand breaks in DNA. Int J Radiat Biol 53:39-48, 1988
2. Veatch W, Okada S: Radiation-induced breaks in DNA in cultured mammalian cells. Biophys J 9:330-346, 1969
3. Warters RL, Hoffer KG, Harris CR, Smith JM: Radionuclide toxicity in cultured mammalian cells. Elucidation of the primary site of radiation damage. Curr Top Radiat Res Q 12:389-407, 1977
4. Yasui LS, Hoffer KG: Role of mitochondrial DNA in cell death induced by ^{125}I decay. Int J Radiat Biol 49:601-610, 1977
5. Sinclair WK: Mechanisms of radiation interaction with DNA. Potential implications for radiation protection. Radiat Res 112:407-417, 1987
6. Teoule R: Radiation-induced DNA damage and its repair. Int J Radiat Biol 51:573-589, 1987
7. Lesko SA, Drocourt JL, Yang SU: Deoxyribonucleic acid-protein and deoxyribonucleic acid interstrand cross links induced in isolated chromatin hydrogen peroxide and ferrous ethylenediamine -tetraacetate chelates. Biochemistry 21:5010-5015, 1982
8. Natarajan AT, Obe G: Molecular mechanisms involved in the production of chromosomal aberrations. I. Utilization of Neurospora endonuclease for the cell cycle. Mutation Res 52:137-149, 1978
9. Natarajan AT, Obe G: Molecular mechanisms involved in the production of chromosomal aberrations. III. Restriction endonucleases. Chromosoma Berlin 90:120-127, 1984
10. Bryant PE: Enzymatic restriction of mammalian cell DNA using Pvu II and Bam HI. Evidence of the double-strand break origin of chromosomal aberrations. Int J Radiat Biol 46:57-65, 1984
11. Kohn KW, Erickson LC, Ewig RAG, Friedman CA: Fractionation of DNA from mammalian cells by alkaline elution. Biochemistry 15:4629-4637, 1976
12. Blocher D, Pohlit W: DNA double strand breaks in Ehrlich ascites tumor cells at low doses of X-rays. II. Can cell death be attributed to double-strand breaks ? Int J Radiat Biol 42:329-338, 1982
13. Dugle DL, Gillespie CJ, Chapmam JD: DNA strand breaks, repair and survival in X-irradiated mammalian cells. Proc Nat Acad Sci 73:809-812, 1976
14. Chadwick KH, Leenhouts HP: The rejoining of DNA double-strand breaks and a model for the formation of chromosomal rearrangements. Int J Radiat Biol 33:517-529, 1978
15. Bradley MO, Kohn KW: X-ray induced DNA double strand break porduction and repair in mammalian cells as measured by neutral filter elution. Nucleic acid Research 7:793-804, 1979
16. Radford IR: The level of induced DNA double-strand breakage correlates with cell killing after X-iradiation. Int J Radiat Biol 48:45-54, 1985
17. Meyn RE, Jenkins T: Variation in normal and tumor tissue sensitivity of mice to ionizing radiation-induced DNA strand breaks in vivo. Cancer Res 43:5668-5673, 1983
18. Koval TM, Kazmar ER: Eluting solution composition affects DNA double-strand break analysis by filter elution. Int J Radiat Biol 54:739-747, 1988
19. Bryant PE: Enzymatic restriction of mammalian cell DNA: Evidence for the double strand breaks as potentially lethal lesions. Int J Radiat Biol 48:55-60, 1985
20. Yamada Y, Neriishi S, Ishimaru T, Shimba N, Hamilton HB, Ohgushi Y: Effects of atomic bomb radiation on the differentiation of B lymphocytes and on the function of Concanavalin A-induced suppressor T lymphocytes. Radiat Res 101:351-355. 1985

= 국문초록 =

γ-Ray 조사에 따른 사람의 정상임파구와 마우스 정상임파구의 DNA Double Strand Break 발생율에 대한 비교분석

원자력병원 치료방사선과*, 방사선의학 연구실

김태환 · 김성호 · 정인용 · 조철구* · 고경환* · 류성렬*

각 방사선량의 조사에 따른 사람 정상임파구, 마우스 정상임파구, 그리고 EL-4 마우스 백혈병 세포의 DNA double strand breaks 발생율에 대하여 비교분석 하였던 바 다음과 같은 결과를 얻었다.

LPS와 PHA를 첨가하여 각각 배양한 마우스 정상임파구의 DNA double strand breaks의발생율을 strand scission factor의 기울기로 비교평가해 본 바 LPS를 첨가배양한 군이 DNA DSB가 더 많이 형성되었다($p<0.005$). 이것으로 볼 때 DNA double strand breaks 발생에 있어서는 B cells이 T cells보다 더 민감하였다. 또한 EL-4 백혈병 세포는 정상 임파구보다 유의하게 DNA DSB가 더 많이 형성된 것이 관찰되었다($p<0.005$).

한편 사람 정상임파구와 마우스 정상임파구 사이의 intrinsic radiosensitivity는 시험관내 실험에서 비교적 유사한 kinetics를 나타냈으나, 마우스 정상임파구가 사람 정상임파구보다 DNA DSB수율이 더 낮았다. 그래서 방사선을 조사하고 3.5 시간이 지난후에 10% DNA DSB 발생에 필요한 선량을 두 군간에 비교평가해 본 바 마우스 정상임파구의 선량이 더 높게 나타났다.

이상의 실험결과를 정확하게 설명하기는 어렵지만 아마도 환경적인 요인과 유전적인 요인 때문인 것으로 사료되었다. 그리고 본 연구결과는 방사선조사에 따른 임상반응의 이해와 cytometric assessment의 방사선 생물학적 parameter로 이용될 수 있을 것으로 판단되었다.