Lack of Recovery from Radiation Damage on Colony Forming Ability and on Membrane Charge in a Burkitt Lymphoma Cell Line

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A Burkitt lymphoma cell line, P3HR-1, was found to be very radiaosensitive ($n=1$, $D_0=67$R) and showed no substantial recovery by dose-fractionation. Measuremant of electrophoretic mobility of whole cells and isolated nuclei after irradiation demonstrated that the loss of negative charge on the membrane was irreversible in this cell line. Analysis of frequency distribution pattern of electrophoretic mobility indicated a good correlation between the loss of colony forming ability and the irreversible loss of membrane charge. The surface saccharides responsible for electrokinetic behavior of the P3HR-1 cells mainly consisted of sialic acid and chondroitine sulfate, but not of hyaluronic acid. The mobility reduction by the removal of these saccharides was reversible and did not result in cell death.

INTRODUCTION

Lymphocytes and lyphatic leukemia cells have been known to be very sensitive to radiation and some of them have exponential dose-survival curves.[1~3] The reasons for the absence of repair of sublethal damage, however, are still unknown. In this experiment, Burkitt lymphoma cells cultured in vitro was used, which exhibited small Do and "n" value of 1. We assumed a defect in repair mechanisms from membrane damage as one of the factors responsible for this lack of recovery, and investigated the electrophoretic behavior of irradiated cells and constituents of cell membrane. The generation time was measured to characterize this cell line.

MATERIALS AND METHODS

Cell line and colony formation

A Burkitt lymphoma cell line designated P3HR-1[4] was used. The cells have been maintained in vitro as suspension culture in Eagle's minimum essential medium containing 20% fetal calf serum. For the assay of colony forming ability, cells were incubated in the medium containing 0.3% agar on the base layer of 0.4% agar medium[5] in a CO_2-incubator. Number of colonies which consisted of more than 50 cells was counted 15 days after irradiation. The plating efficiency was between 40 to 60%.

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Electrophoresis

The electrophoretic mobilities of individual cells (or noclei) were measured at 25 ± 0.5°C in an apparatus of the type described by Fuhrmann and Ruhenstroth-Bauer.[6] Each cell was allowed to move 15 μ alternately in both directions following reversal of current in 1/15 M phosphate buffer supplemented with 5.4% glucose (pH 7.3, ionic strength 0.167). The mobility of cells was determined by three separate experiments on 100 to 200 cells for each point and calculated in μ/sec per V/cm. Inorder to check the reproducibility of the apparatus, the mobility of normal rat erythrocytes was measured before each experiment. Under the above conditions, the mobility of rat erythrocytes was $-1.100 \pm 0.040\ \mu.\ sec^{-1}.\ V^{-1}.\ cm.$[7]

Preparation of isolated nuclei

Cells were homogenized with Waring blendor for 20 seconds in 15 m*l* of ice-cold 1.0% citric acid supplemented with 0.2 mM $CaCl_2$ and 0.2 mM $MgCl_2$. Nuclei were sedimented by centrifugation for 3 minutes at 600 xg, and then washed with 1/15 phosphate buffer added with 5.4% glucose. All the procedures were carried out at 0°C. The isolated nuclei were examined by electron microscope and found to retain only the inner nuclear membrane as reported by Gurr et al.[8] For the measurement of mobility, clean nuclei without cyroplasmic fragments were chosen under a phase contrast microscope.

Enzymatic treatment

Cells were treated with neuraminidase from *Vibrio cholerae* (General Biochemicals, Tnc., Ohio), Chondroitinase-ABC[9] (Seikagaku Kogyo Co., LTD., Tokyo) and hyaluronidase from *Streptomyces hyalurolyticus nov. sp.*[10] (Seikagaku Kogyo Co., LTD, Tokyo) at the concentration of 20 units/m*l*, 1 unit/m*l* and 10 TRU/m*l*, respectiuely. About 1×10^7 lymphoma cells were suspended in 1 m*l* of enzyme solution and incubated at 37°C for 30 min. The buffer solutions used were Hanks balanced salts solution (pH 7.0) for neuraminidase, 0.05 M Veronal buffered saline (pH 8.0) containing 0.1% albumine for chondroichinase ABC, and 0.02 M Veronal buffered saline (pH 5.0) for hyaluronidase. The enzyme-digested cells were washed twice with the phosphate buffer, and their electrophoretic mobilities were then compared with that of control cells similarly treated in the corresponding buffers without the enzyme.

X-irradiation

Irradiation of cell suspension in a culture dish was carried out with 200 KVp X-rays in room temperature (15–20°C). The physical factors of exposure were 200 KVp, 20 mA, 0.5 mmAl+0.5 mmCu filter added, half value layer of 1.13 mmCu, 40 cm target-sample distance and 96 R/min.

RESULTS

Dose survival relation on colony formation

Surviving fractions of the cells were plotted on a semilog scale against exposure in Fig. 1 (a). This dose-survival curve did not have very substantial shoulder and appeared to be "single-hit" type. The formula of the curve was obtained by the method of least squares as $\log Y = -0.0062\,X - 0.001$, Where Y represents the surviving fraction, and X refers to expsure in R. On this formura surviving fraction at 0 R, that is "n" value, was 0.998. The dose required to make the survival reduced to 37% of control was 67 R.

Dose fractionation test

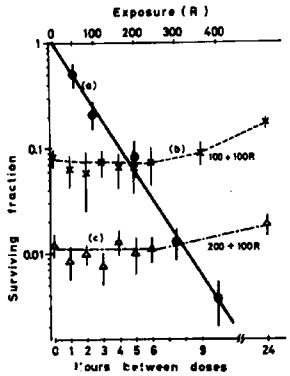

Fig. 1. Curve (a) shows single dose-survival relation on colony formation (n=1, D_{37}=67 R). In split-dose experiment cells were irradiated with 100R (curve b), or 200R (curve c) and 100R with various time intervals.

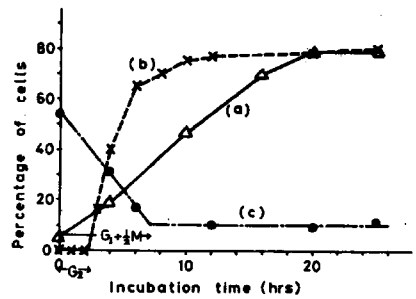

Fig. 2. (a) Accumulation of mitotic cells/total cells, (b) labeled mitoses/total mitoses and (c) unlabeld cells in interphase/total cells after the simultaneous administration of ^{3}H-thymidine 0.1 μCi/ml and colcemide 0.1 μg/ml.

Cell samples were exposed to doses of 100R and additional 100R, or 200R and 100R separated by varying intervals to examine if Burkitt lymphoma cells had an ability to repair the damage between fractionated doses. Cells were suspended in culture medium at 37°C during the time interval. In Fig. 1 (b) and (c) survival fractions were plotted as a function of time between the two exposures. In both conditioning doses almost constant survivals were demonstrated without reference to time intervals from 1 to 6 hours. About twofold survival was obtained by the dose fractionation of 24 hour interval. Mitotic delay was 3 and 7 hours after irradiation of 100R and 200R respectively by the measurement of the accumulation of mitotic cells with colcemide.

Generation cycle of the cell line

The doubling time of the logarithmically growing cells was about 22 hours during the cell concentrations from 1.5 to 8.0×10^5 cells/ml. At this actively growing state, less than 1% of cells bore Epstein-Barr virus antigen determined by the indirect immuroflurescence method.[11] Simultaneous administration of ^{3}H-thymidine 0.1 μCi/ml and colcemid 0.1 μg/ml to log-phase cells resulted in the accumulation of labeled and unlabeled mitotic cells as shown in Fig. 2. Accumulation of mitotic cells with time by colcemid (curve a) indicated that the generation time was about 20 hours. The length of G_2-phase was directly estimated[12] at 2.5 hours from the result on labeled mitoses/total mitoses (curve b). According to the method of Puck and Steffen[12] and Maekawa and Tsuchiya,[13] the length of G_1+1/2 M phase was estimated at 7.5 hours from curve C (unlabeled cells in interphase/total cells). The fraction of dormant cells was estimated at 10% from curve c. Mitotic index at 0 hr-culture was 2.8%, and the labeling index after 30 min incubation with ^{3}H-thymidine was 51% on autoradiographs.

To ascertain the generation time, cell growth was synchronized with hydroxyurea and colcemid. After the preincubation with 0.5 mM hydroxyurea for 14 hours, washed cells were then incubated with colcemid (0.1 μg/ml) for the next 11 hours. After the removal of colcemide, the second peak of mitotic index appeared 19.5 hours after the first peak. ^{3}H-thymidine incorporation into DNA decreased to minimum 8 to 10 hours

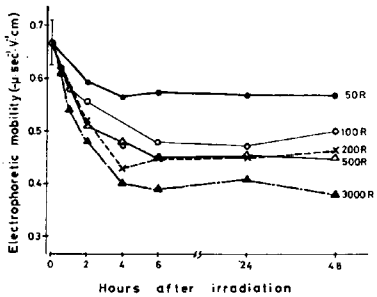

Fig. 3. Irreversible decrease in the electrophoretic mobility of Burkitt cells P3HR-1 with time after the irradiation with various doses.

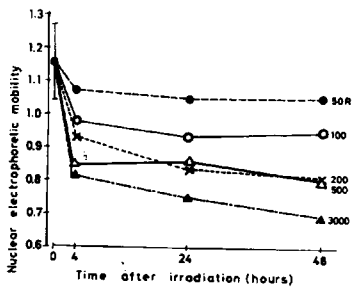

Fig. 4. Change in the electrophoretic mobility of nuclei isolated at various time after x-irradiation.

after removal of hydroxyurea from culture medium.[14] From these results, generation time was estimated at 19.5 hrs, M-phase 0.7 hours, G_1-phase 7.1 hours, S-phase 9 hours and G_2-phase 2.7 hours.

Change in cellular or nuclear electrophoretic mobility following irradiation

The mean electrophoretic mobility and standard deviation of non-treated P3HR-1 cells was $-0.677 \pm 0.045\ \mu.\ sec^{-1}.\ V^{-1}.\ cm$. As indicated in fig. 3, reduction in the mobility was observed during the first 4 hours after irradiation. Irradiation with larger doses produced a more profound and more rapid decrease in the negative net charge of the cell surface. About 32% of negative charge was lost during the first 2 hours after irradiation with 3000R. There appeares no substantial recovery in electrophoretic mobility for 48 hours after irradiation even with 50R.

Figure 4 shows the electrophoretic mobility of nuclei isolated at various time after irradiation. The figure is almost the same as that of cellular electrophoresis in figure 3. Dose-dependent reduction and no recovery in nuclear mobility were observed after irradiation.

In a frequency distribution of cellular electrophoretic mobility, heavily irradiated cells showed a completely separate distribution from that of non-irradiated cells.[19] Irradiation with small doses produced two peaks in the frequency distribution of mobility. And the mobility value of each peak corresponded to the peak mobility in non-irradiated cells and irradiated with 3000R, respectively.[19] The similar result was obtained in the frequency distribution of nuclear electrophoretic mobility. The fraction of electrokinetically intact cells or nuclei 4 hours after irradiation were plotted as a function of dose in fig. 5. The correlation coefficient (r) was 0.98 between surviving fraction on colony-forming ability and the fraction of electrophoretically intact cells. The correlation coefficient was 1.009 between colony survival and intact fraction on nuclear electrophoresis.

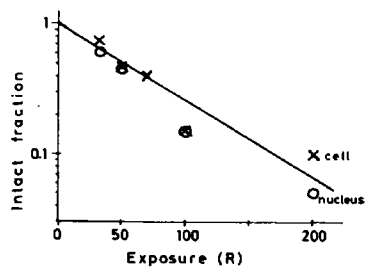

Fig. 5. Relationship between x-ray dose and the fraction of electrophoteti cally intact cells (x) or intact nuclei (o). The line represents dose-survival relation on colony formation.

Changes in electrophoretic mobility by enzymatic treatments

To investigate the anionic saccharides of the cell surface responsible for elestrokinetic behavior,

Table 1. Decrease in electrophoretic mobility of Burkitt lymphoma cells after the treatment with each enzyme for 30 minutes (mean ± standard deviation)

Enzyme	Treated	Nontreated	% decrease
Neuraminidase 20 units/m*l* (10^7 cells)	-0.149 ± 0.046	-0.641 ± 0.070 (μ. sec^{-1}. V^{-1}. cm)	76.7%
Chondroichinase ABC 1 unit/m*l* (10^7 cells)	-0.324 ± 0.071	-0.655 ± 0.094	38.8
Hyaluronidase 10 TRU/m*l* (10^7 cells)	-0.651 ± 0.063	-0.653 ± 0.063	3.1

the mobility was measured after treatment with three kinds of enzymes (Table 1). Removal of sialic acid from cell surface by neuraminidase resulted in 77% desrease in the electrophoretic mobility, and that of chondroitinase-ABC resulted in 49% decrease. On the other hand, treatment with streptomyces hyaluronidase had no effects on the mobility. When cells were re-incubated in the culture medium after the removal of the enzymes, the electrophoretic mobility recovered very slowly during the next 24 hours. The cell viability by means of dye exclusion test was not lost after the enzymatic treatment.

DISCUSSION

Since the original report of Elkind and Sutton,[15] recovery of radiation damage after the first dose has been reported for a number of different cell systems.[16] The present study suggests that in a Burkitt lymphoma cell line P3HR-1 there is little, if any, sublethal damage repaired within a time-interval between fractionated doses. An increase in surviving fraction by cell proliferation[17,18] was demonstrated. In this cell line, there appeared no recovery in the electrokinetic behavior, and close correlation was found detween the loss of colony forming ability and the loss of negative charge of cell surface or nuclear surface 4 hours after irradiation. On the other hand, a melanoma cell line B16-C2W and a mammary carcinoma cell line FM3A, which showed recovery in dose-fractionation experiment, also showed recovery in electrophoretic mobility after x-irradiation with small doses up to 500R.[23] Analysis of frequency distribution pattern of cell electrophoretic mobility 24 hours after irradiation demonstrated that the fraction of electrophoretically recovered cells corresponded to the surviving fraction.[23]

It has been reported that negative charge of cells is largely dependent on carbohydrate-rich layer of cell surface.[20] This layer contains not only sialic acid but also acid mucopolysaccharide-protein complexes as negatively charged substances in many cell types.[21] Decrease in the mobility after the enzymatic treatment suggestes that sialic acid and chondroitine sulfate are main substances responsible for electrokinetic behavior of the Burkitt lymphoma cell line, and hyaluronic acid is not; although the percentage of reduction in mobility dose not directly mean the relative quantity of each saccharides on the cell surface. The mobility reduction by enzymatic treatment was reversible and did not result in the cell death.

Further investigation is required how irreversible loss of membrane charge could relate with loss of colony forming ability. The complex of nuclear membrane and DNA[22] or chromosome might be a critical target of x-irradiation.

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