Analysis of the Factors in Determining Radiosensitivity in Mammalian Cells by Using Radio-Sensitive and -Resistant Clones Isolated from HeLa S3 Cells in Vitro

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(Received May 10, 1976; Revised version received June 7, 1976)

The factors in determining radiosensitivity of cultured mammalian cells were analysed by using two clones each having different radiosensitivities. The radiosensitive clones were isolated from HeLa S3 cells by the N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-treatment, X-irradiation (200 R) and 5-bromodeoxyuridine (B UdR)-visible light method. On the other hand, the radioresistant clone was isolated by single X-irradiation (2000 R) from MNNG-treated HeLa S3 cell population. The radiosensitivities expressed in D0 and DQ values were 110 and 140 R in radiosensitive SM-1a clone and 180 and 230 R in radioresistant RM-1b clone respectively. The biological and biochemical characteristics of both clones such as the distribution of chromosome numbers, formation and rejoining of single strand breaks in DNA caused by X-irradiation, non-protein sulphhydryl (NPSH) and apparent total sulphhydryl (APSH) contents were measured. Among the characteristics analysed, different contents of NPSH in the cells were well correlated to their radiosensitivities among the original HeLa S3 cells, SM-1a and RM-1b clones. Additionally, it was found that the radioresistant L-P3 Co-3 cells isolated by Tsuboi et al. from the original mouse L-P3 cells by means of serial irradiation with 60Co γ-rays have more abundant NPSH than the original L-P3 cells. From these results, it can be concluded that the amount of NPSH play the main role in determining radiosensitivity in cultured mammalian cells.

INTRODUCTION

The development of radioresistant tumors in the course of radiation therapy has prompted to many efforts in isolating radioresistant cell lines in vitro. The radioresistant cell lines have been isolated from various cultured cell lines by irradiation with X-rays2,3 or γ-rays3,4 and continuous irradiation with β-rays5 emitted from tritium which was contained in culture medium. On the other hand, a cell line being more radiosensitive than the original L5178Y cells was fortuitously isolated after single irradiation with a small dose of X-rays.3 These cells, different in terms of radiosensitivity have been thought to be the useful tools for finding the factors in
determining radiosensitivity in cultured mammalian cells. In regard to the sensitivity of mammalian cells to ultraviolet light (UV), the introduction of the cells obtained from patients with genetic disease, Xeroderma pigmentosum and experimentally isolated S-2M cells, which were deficient in excision ability of pyrimidine dimers, led to a significant advance in our knowledge on the repair mechanisms in UV-damaged cellular DNA.

On the other hand, various biological characteristics such as chromosome numbers, reparable of X-ray-induced DNA damage, growth rate; and sulfhydryl contents in the cells have been suggested as the factors influencing the radiosensitivity in mammalian cells. However, the main factor for determining the cellular radiosensitivity is still unknown. In order to know this, it is necessary to isolate the stable radio-resistant and -sensitive clones from an original cell line and to compare the biological characteristics in these clones. In the present study, we have analysed biological characteristics of clones having different sensitivities to X-rays which were isolated from the original HeLa S3 cells, and have found that non-protein sulfhydryl contents in each clone play a main role in determining their radiosensitivities.

MATERIALS AND METHODS

Cells and medium

HeLa S3 cells cultured in TD-40 or Roux culture bottles containing a culture medium consisted of 90% Eagle's MEM (Nissui Seiyaku Co. Ltd., Tokyo) and 10% bovine serum were used in the present study.

Isolation of radiosensitive clones from the original HeLa S3 cells

Radiosensitive clone was isolated from the original HeLa S3 cells with the application of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), small doses of X-rays, and 5-bromodeoxyuridine (BUdR) and visible light exposure, which is a slight modification of the procedures originally described by Puck et al.

Exponentially growing 3 × 10^6 HeLa S3 cells were inoculated into two Roux culture bottles and incubated for 24 hours at 37°C. The cells in one Roux bottle were treated with 0.5 μg/ml of MNNG for 24 hours; by this procedure the surviving fraction of the cells was reduced to 0.2. The cells were rinsed twice with fresh medium and incubated for 9 more days. Then, the cells in Roux culture bottle were harvested by trypsinization and suspended into fresh medium. Ten ml aliquots of the cell suspension (10^6 cells/ml) were poured into short pyrex test tubes (inner diameter 1.2 cm) and irradiated with various doses of X-rays, up to 400 R, at a dose rate of 75 R/min in air as measured with a Victoreen condenser chamber. The cells in each test tube were centrifuged immediately after the irradiation and resuspended into 10 ml of medium (free of phenol red) containing 10^-6 M BUdR and incubated in a TD-40 culture bottles for 4 days. After replacing with Eagle's MEM medium free of phenol red, cells were exposed to visible light for 2 hours. This was performed by exposing
the cells attached in a monolayer on inner surface of TD-40 culture bottles containing white Eagle's MEM medium to a depth of approximately 1.8 mm, to 60 W light (six 10-W fluorescent lamps, National "Cool White" tubes, 30 cm long), i.e. two TD-40 culture bottles placed on the glass plate of 1.5 mm thick were exposed to light from three lamps each, which were fixed at a distance of 5 cm from the upper and lower surface of the glass plate. After exposure, the medium was replaced with fresh growth medium and the culture bottles were incubated for further three weeks until clones develope to appreciable size. On the other hand, the cells in Roux culture bottle defined as control were harvested 5 days after the first cell inoculation and processed in the same way as mentioned for MNNG-treated cells.

In MNNG-treated cell population, seven clones appeared in the bottle after 200 R-irradiation and subsequent treatment with BuDR-visible light. One clone each appeared in the bottles which were irradiated with 300 and 400 R respectively. Working from the assumption that the lower doses of X-rays were appropriate for obtaining radiosensitive clones, we did not use these two clones obtained by irradiation with more than 300 R of X-rays for the following experiments. The seven clones which appeared after 200 R-irradiation were separately isolated and designated SM-1a, SM-1b, SM-1c, SM-1d, SM-1e, SM-1f and SM-1g cells, as shown in Table 1.

On the other hand, in MNNG-untreated cell population, four clones appeared in a culture bottle after 200 R-irradiation and subsequent treatment with BuDR-visible light. These four clones were separately isolated for the following experiments.

**Isolation of radioresistant clones from the original HeLa S3 cells**

Exponentially growing HeLa S3 cells (3×10⁶ in number) were inoculated into each

### Table 1

| Number of cells inoculated per Roux bottle | Concentrations of MNNG administered for 24 hours (µg/ml) | Culture days till harvesting after administration with MNNG (days) | Number of cells harvested per Roux bottle | Doses of X-rays irradiated to 10⁶ cells (R) |
|------------------------------------------|----------------------------------------------------------|---------------------------------------------------------------|------------------------------------------|------------------------------------------|
| 3.0×10⁶                                  | 0.5                                                      | 9                                                             | 2.54×10⁷                                 | 0                                        |
|                                          |                                                           |                                                                |                                          | 100                                      |
|                                          |                                                           |                                                                |                                          | 200                                      |
|                                          |                                                           |                                                                |                                          | 300                                      |
|                                          |                                                           |                                                                |                                          | 400                                      |
|                                          | 0                                                         | 3                                                             | 0.93×10⁷                                 | 0                                        |
|                                          |                                                           |                                                                |                                          | 100                                      |
|                                          |                                                           |                                                                |                                          | 200                                      |
|                                          |                                                           |                                                                |                                          | 300                                      |
|                                          |                                                           |                                                                |                                          | 400                                      |

*: Visible light
Roux culture bottle and incubated for 24 hours at 37°C. Then, the cells were treated with 0.5 μg/ml of MNNG for another 24 hours. After cultivation in a normal growth medium for 9 days, the cells were harvested and resuspended into fresh culture medium at a concentration of 10⁶ cells/ml. Ten ml aliquots of the cell suspension were poured into short pyrex test tubes (inner diameter 1.2 cm) and irradiated with either 1500 or 2000 R of X-rays, and a dose rate of 115 R/min in air. One (10⁶ cells) or 10 ml (10⁷ cells) of irradiated cell suspensions were distributed into TD-40 or Roux culture bottles containing 9 or 40 ml of fresh culture medium respectively, and incubated at 37°C for three weeks until clones develop to appreciable size. The MNNG-untreated cells in three Roux culture bottles designated as control were harvested 5 days after the first cell inoculation and processed in the same way as described for MNNG-treated cells.

In MNNG-treated cell population, as shown in Table 2, 13 clones appeared in a culture bottle inoculated with 10⁶ cells irradiated with 1500 R of X-rays and 4 clones appeared in a bottle inoculated with 10⁷ cells irradiated with 2000 R three weeks after irradiation. However, no clone appeared in a bottle inoculated with 10⁶ cells irradiated with 2000 R. On the other hand, in control cell population, 10 clones appeared in a bottle inoculated with 10⁶ cells irradiated with 1500 R and one clone appeared in a bottle inoculated with 10⁷ cells irradiated with 2000 R.

Five clones (RM-1a, RM-1b, RM-1c, RM-1d and R-1 cells) obtained from either MNNG-treated or -untreated cell populations after irradiation with 2000 R of X-rays were used for the following experiments, with the simple assumption that the cells which survived the larger doses of X-irradiation might have radioresistant type.

| Culture days in 10⁻³M BUdR | Exposure time to visible light (60 W) | Number of colonies formed per bottle 3 weeks after V.L. exposure | Cell names designated |
|-----------------------------|--------------------------------------|---------------------------------------------------------------|----------------------|
| (days)                      | (hours)                              | 0                                                             |                      |
| 4                           | 2                                    | 0                                                             | SM-1a, -1b, -1c, -1d, -1e, -1f and -1g |
| 4                           | 2                                    | 0                                                             | S-1a, -1b, -1c and -1d |

for obtaining radiosensitive cells from the original HeLa S3 cells
Table 2

| Number of cells inoculated per Roux bottle | Concentrations of MNNG administered for 24 hours (µg/ml) | Culture days till harvesting after administration with MNNG (days) | Number of cells harvested per Roux bottle |
|------------------------------------------|--------------------------------------------------------|---------------------------------------------------------------|------------------------------------------|
| 3.0 × 10⁶                               | 0.5                                                   | 9                                                             | 2.07 × 10⁷                               |
| 3.0 × 10⁶                               | 0                                                     | 3                                                             | 0.90 × 10⁷                               |

**Determination of sensitivity of cells to X-rays by colony-forming method**

Exponentially growing cells of each isolated clone were harvested by trypsinization and suspended into fresh medium. Five ml aliquots of the cell suspension (400 cells/ml) were poured into short test tubes and irradiated with various doses of X-rays, at a dose rate of 75 R/min in air. After each 1 ml of unirradiated or irradiated cell suspension was distributed into glass Petri dishes (diameter 5 cm) and supplemented with an additional 4 ml of culture medium, the dishes were incubated in humidified 5% CO₂ incubator for 14 days. The number of colonies containing more than 50 cells were counted after Giemsa staining.

**Analysis of chromosome numbers**

The cells in the logarythmic phase of growth were treated with colchicine at a final concentration of 10⁻⁵ M for 6 hours. Then, the cells were collected, and washed twice with Hanks’ balanced salt solution. After this, cell preparations for chromosome study were obtained by an air-dry method and Giemsa staining.

**Analysis of the formation and rejoining of single strand breaks in X-irradiated cellular DNA by alkaline sucrose gradient method**

The cells labeled with 1 µCi/ml of ³H-thymidine (5 Ci/mM) for 24 hours were harvested by trypsinization, resuspended into fresh medium and irradiated with 5 and 10 KR of X-rays, at a dose rate of 450 R/min in air, in an ice bath. After various times of post-irradiation incubation at 37°C, cells were washed twice with ice cold phosphate buffer saline (PBS) and resuspended into PBS at a concentration of 1×10⁵ cells/ml.

Each 4.8 ml of linear 5-20% sucrose gradient containing 0.1 N NaOH, 0.9 M NaCl and 0.003 M Na₂EDTA was prepared in a 5 ml butylate tube. Immediately before the cells were added, 0.25 ml of lytic solution containing 0.45 N NaOH, 0.5 M NaCl and 0.01 M Na₂EDTA (pH 13.1) were carefully layered on the top of each gradient. Then,
for obtaining radioresistant cells from the original HeLa S3 cells

| Doses of X-irradiation (R) | Number of cells irradiated | Number of colonies formed per bottle 3 weeks after irradiation | Cell names designated |
|---------------------------|----------------------------|---------------------------------------------------------------|----------------------|
| 1500                      | $10^6$                     | 13                                                            | RM-1a, 1b, 1c        |
| 2000                      | $10^6$                     | 0                                                             |                      |
| 2000                      | $10^7$                     | 4                                                             | RM-1d                |
| 1500                      | $10^6$                     | 10                                                            |                      |
| 2000                      | $10^6$                     | 0                                                             |                      |
| 2000                      | $10^7$                     | 1                                                             | R-1                  |

0.025 ml (3000-5000 cells) of cell suspension in ice cold PBS were layered upon the lytic solution and these preparations were kept at 25°C for 5 hours. After centrifugation using a Beckman SW-50L rotor (36,000 rpm for 60 min at 12°C), 20-drop fractions were collected onto Whatman 3MM paper disks and the disks were washed with 5% ice cold trichloroacetic acid and dried. The radioactivity of each disk was counted in Packard liquid scintillation counter. The gradients were calibrated with $^{3}C$-labeled λ phage DNA (provided by Dr. T. Andoh, The Institute of Medical Science, University of Tokyo) and the sedimentation coefficients ($S$) were estimated by the Burgi and Hershey's equation.

Determination of non-protein sulfhydryl and apparent total sulfhydryl contents in the cells

For determining the non-protein sulfhydryl (NPSH) content in cultured cells, a slight modification of the method described by Ohara and Terasima was used. The monolayered cells were carefully washed once with PBS and suspended in a small volume of PBS. After centrifugation, the cell pellets were suspended into a small volume of ice cold 5% sulfosalicylic acid and allowed to stand in the ice bath for 90 min. The acid extracts were obtained by centrifugation at 7000 g for 15 min, and brought to pH 6.8 by adding PBS containing 8 N NaOH. Immediately after addition of 0.1 ml 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 36.9 mg was dissolved in 100 ml of PBS, pH 7.0) to 5 ml of the extracts, optical density at 410 nm was measured by Hitachi spectrophotometer Type 102. The solution of reduced glutathione at an appropriate concentration was used as a standard in every experiment. The content of NPSH was calculated by using the Ellman's equation. For measuring apparent total sulfhydryl (APSH) content, cells were dissolved in 0.1 N NaOH solution in the ice bath. The cell lysate was neutralized with a equal volume of 0.1 N HCl and diluted with appropriate volumes of PBS (pH 6.8). Five ml of this neutralized solution were rapidly reacted with 0.1 ml DTNB as mentioned above.
On the other hand, protein content of cultured cells was determined by the method of Lowry et al.\textsuperscript{20} with a slight modification.

\textbf{RESULTS}

The establishment of radio-sensitive and -resistant clones

The sensitivities to X-rays of various clones isolated were assayed by colony-forming method after being cultured for three months from the time of isolation. By

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig_1}
\caption{X-rays dose-survival curves of various clones isolated from the original HeLa S3 cells. The survival curves were fitted by eyes along the means of surviving fractions to each dose of X-rays obtained from three independent experiments.}
\end{figure}
this time, all clones retained almost the same growth rates as that of the original HeLa S3 cells.

As shown in Figure 1, only SM-1a clone among seven clones obtained by the MNNG-200 R of X-rays-BUdR-visible light method was sensitive to X-rays. In addition, S-1a, S-1b, S-1c and S-1d clones obtained from MNNG-untreated cell population showed the similar radiosensitivities seen from the original HeLa S3 cells.

On the other hand, RM-1b clone among five clones isolated from MNNG-treated and -untreated cell populations after 2000 R-irradiation was the most radioresistant, as

![X-rays dose-survival curves of various clones isolated from the original HeLa S3 cells. The survival curves were fitted by eyes along the means of surviving fractions to each dose of X-rays obtained from three independent experiments.](image-url)
shown in Figure 2. Two other clones (RM-1c and R-1) showing intermediate radioresistance were also isolated for the subsequent experiments, these two clones gradually lost their radioresistance in subsequent culture and finally showed almost the same radiosensitivity as the original HeLa S3 cells. But the radiosensitivity of RM-1b clone remained stable during the subculture.

Six months after their isolation, both radiosensitive SM-1a and radioresistant RM-1b cells were subjected to the second determination of their radiosensitivities. Their radiosensitivities quoted by D₀ and D⁰ values were shown in Figure 3. It is

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**Fig. 3.** Survival curves of SM-1a and RM-1b cells irradiated with various doses of X-rays. The survival curve of each clone was obtained from eight independent experiments. Vertical bars show standard deviations.
clear that both $D_0$ and $D_q$ values in RM-1b cells were about 1.6 times larger than those in SM-1a cells.

**Number of chromosomes in radio-sensitive and -resistant clones**

The number of chromosomes in SM-1a and RM-1b cells were investigated 6 months after their isolation. The chromosome numbers in the original HeLa S3 cells

![Graph showing chromosome number distribution for Original HeLa S3 cells, SM-1a cells, and RM-1b cells.](image-url)

**Fig. 4.** Distribution of chromosome numbers in the original HeLa S3, SM-1a and RM-1b cells. The number of chromosomes were determined by examining 100 cells in each clone.
were widely distributed from 57 to 72, having prominent peak of 68 chromosomes as shown in Figure 4. SM-1a cells showed a rather narrower distribution of chromosome numbers and their modal chromosome number was 66. On the other hand, the wide distribution of chromosome number was observed in RM-1b cells. Their modal chromosome number was 62. It was noticed, however, that the cells having a small number of chromosomes which were scarcely observed in the original HeLa S3 cells, increased in RM-1b cells.

Induction of single strand breaks in DNA of SM-1a and RM-1b cells by X-irradiation and their rejoining

SM-1a and RM-1b cells were irradiated with 5 or 10 KR of X-rays and the cells irradiated with 5 KR were incubated in fresh medium at 37°C for 15 and 30 min. The results of these experiments are shown in Figure 5. The sedimentation profiles of the DNA from both unirradiated clones (120 S) are shown in this figure as the controls (Fig. 5A). Immediately after irradiation with 5 or 10 KR of X-rays, significant decrease in sedimentation rates of DNA in SM-1a and RM-1b cells were similarly observed (Figs. 5B and 5C). However, when the cells irradiated with 5 KR of X-rays were incubated at 37°C for 15 and 30 min, the increase in sedimentation rates of DNA in both clones were observed, and the position of the peaks in sedimentation profile of DNA approached those of unirradiated controls (Figs. 5D and 5E). From these observations, it is obvious that not only the formation of single strand breaks in cellular DNA following X-irradiation but also the rates of rejoining of these breaks in DNA during the incubation for 30 min were not significantly different in both SM-1a and RM-1b cells.

The content of non-protein sulfhydryl (NPSH) in SM-1a and RM-1b cells

The content of NPSH in SM-1a and RM-1b cells were determined at various days of culture. For this experiment, the cells were usually subcultured every 4 days. As shown in Figure 6, the changes of NPSH content per cell in both clones during 7 days of cultivation were essentially the same. At the second day of culture, both cells contained maximum amount of NPSH. However, RM-1b cells contained about a 1.5 times greater amount of NPSH than that in SM-1a cells. The NPSH content in both clones decreased thereafter and reached the same level at the 7th day of culture. It is noteworthy that the cells used for determining their radiosensitivities by colony-
forming method were obtained from 2 day-culture in both clones.

**DISCUSSION**

The factors in determining the radiosensitivity in cultured mammalian cells have been investigated mainly in two biological systems, i.e. the analyses of biological characteristics in the experimentally isolated radioresistant cells and the original cell line, and analyses of biological characteristics in the various cell cycle phases showing different sensitivities to X-rays. In this study, the isolation of both radio-resistant and -sensitive cells from the original HeLa S3 cells were tried, since the analysis of biological characteristics in cell lines showing different radiosensitivities was thought to be useful for finding the factors which are responsible for determining their radiosensitivities.

Fig. 6. Changes in the content of non-protein sulfhydryls (NPSH) in SM-1a and RM-1b cells during 7 days of culture. Vertical bars show standard deviations of the mean for five independent determinations.
The procedure for obtaining radiosensitive clone, consisting of various doses of X-irradiation and BUdR-visible light treatment was applied to the cell populations cultured for 3, 6 and 9 days after MNNG-treatment. However, no clone appeared in both 3 and 6 day-cultured cell populations, but several clones were recovered from the cell population cultured for 9 days after MNNG-treatment. The colonies appeared in the culture bottles containing 10⁶ cells from the 9 day-cultured population after irradiation with more than 200 R of X-rays and subsequent treatment with BUdR-visible light. The maximum number of colonies was obtained in the cultures which had been both treated and not treated with MNNG after 200 R of X-rays (Table 1). It is suggested that 200 R of X-rays may be the dose diverging the survival curve of the original HeLa S3 cells to sublethal and lethal portion. These findings also seem to suggest that 200 R is a dose suitable for distinguishing the induced radiosensitive cells from the original HeLa S3 cells unaffected by the MNNG treatment, which were subsequently exposed to BUdR-visible light.

Among the clones isolated, only SM-1a clone obtained by X-irradiation with 200 R was significantly more radiosensitive than the original HeLa S3 cells (Fig. 1). All other clones showed radiosensitivities similar to that of HeLa S3 cells, despite the fact that they could survive BUdR treatment followed by visible light exposure. Two possible explanations may be considered to account for the appearance of these pseudo-radiosensitive clones. First, they were the clones unable to incorporate exogenous BUdR into their DNA, due to either the alteration of membrane permeability or the deficiency of thymidine kinase phosphorylating BUdR to deoxyribonucleotide. Second, they were the clones which behaved exactly similar to radiosensitive clone at the time of X-irradiation with 200 R but retained normal sensitivities to X-rays 3 months after their isolation.

It is known that cultured mammalian cells are unable to discriminate between BUdR and thymidine as a precursor for their DNA synthesis. Examined by the autoradiography, these 7 clones (SM-1a, -1b, -1d, -1e, -1f, S-1a and -1b) were able to incorporate ³H-thymidine into their DNA. This may suggest that these pseudo-radiosensitive clones could escape from the BUdR treatment followed by visible light exposure and survived thereafter, because of the second possibility.

In order to isolate radioresistant clones from the original HeLa S3 cells, a large dose (2000 R) of X-ray was irradiated to 10⁷ cells either treated or untreated with MNNG. In contrast to the case of isolating radiosensitive clones, various clones showing the high resistance and the intermediate resistance to X-irradiation were obtained (Fig. 2). Only RM-1b clone showed a stable radioresistance during the experiments. However, RM-1c and R-1 clones were finally excluded from further experiments because of their unstable radiosensitivities. The instability of radioresistance in these RM-1c and R-1 clones may also be due to the epigenetic changes. Hence, both stable radiosensitive SM-1a and stable radioresistant RM-1b clones (Fig. 3) were used for further experiments.

Courtenay has isolated two stable radioresistant cell lines from mouse leukemic
L5178Y cells by continuous irradiation with \( \beta \)-rays emitted from tritium. One of them showed a wide shoulder in their dose survival curve (increased \( D_0 \) value), while the other showed an increased \( D_0 \) value. These observations moved the researchers to study the repair capacity in radio-resistant and -sensitive cells after X-irradiation. Fox et al.\(^{10} \) could not find any differences in the formation of single strand breaks in DNA of both radioresistant and the original L5178Y cells irradiated with 10 KR of X-rays, and in their rejoining rate during post incubation time.

In the previous experiment, we have compared the numbers of single strand breaks induced in the DNA of various cell lines having \( D_0 \) values from 155 to 180 R.\(^{15} \) However, no significant differences in the number of breaks produced and in the rate of their rejoining were observed among them as well. In the present study, again SM-1a and RM-1b cells also did not show any significant differences in the formation of single strand breaks in their DNA after 5 and 10 KR-irradiation with X-rays, and in the rate of rejoining during postincubation time following 5 KR-irradiation (Fig. 5).

Since the clear relationship between nuclear volume and radiosensitivity in higher plants was demonstrated by Sparrow et al.,\(^{23} \) the correlation between the amount of genetic materials (number of chromosomes or DNA contents) in the cells and radiosensitivity has been extensively studied in mammalian cells. It has been frequently observed that the radioresistant cells which appeared after repeated irradiation with X- or \( \gamma \)-rays had a smaller number of chromosomes than the original cell lines.\(^{4,9,24,25} \) However, Till\(^{26} \) was unable to demonstrate the differences in radiosensitivity among the L cell variants having different chromosome numbers, and the radioresistant cell lines isolated by Courtenay\(^{5} \) had the same range of chromosome numbers as the original cell line.

In this study, the modal chromosome numbers in the original HeLa S3, SM-1a and RM-1b cells were 68, 66 and 62 respectively. On the other hand, \( D_0 \) values obtained from the dose-survival curves were 160, 110 and 180 R in the original HeLa S3, SM-1a and RM-1b cells respectively. Therefore it is not possible to explain the differences in radiosensitivity of these three cell lines by the differences in their modal chromosome numbers.

On the other hand, the amount of NPSH in radioresistant RM-1b cells was greater than that in radiosensitive SM-1a cells during the first 4 days of culture (Fig. 6). The ratios of NPSH content in the original HeLa S3 and RM-1b cells to that in SM-1a cells at the second day of culture were 1.1 and 1.5, respectively. Incidentally, the ratios of \( D_0 \) and \( D_0 \) values of the other two cell lines to those in SM-1a cells were 1.5, 1.1 in the original HeLa S3 and 1.6, 1.6 in RM-1b cells respectively. Thus, the cellular content of NPSH was well correlated to the radiosensitivities in three cell lines used for the present study. In contrast to this, we could not observe any significant differences in the amount of apparent total sulfhydryls (APSH) among these three cell lines as shown in Table 3.

In order to confirm the relationship between the radiosensitivity and NPSH con-
tent of the cells, mouse L·P3 and L·P3 Co-3 cells obtained by courtesy of Prof. Katsuta were also used. L·P3 Co-3 cells were isolated by serial irradiation of $^{60}$Co $\gamma$-rays with total dose of 3500 R from the original L·P3 cells which were able to grow in protein and lipid free medium. We adapted both L·P3 and L·P3 Co-3 cells to Eagle's MEM medium which was supplemented with 10% bovine serum in this laboratory, in order to promote their growth.

The radiosensitivities of these two cell lines were examined by the colony-forming

![Graph showing survival curves](image)

**Fig. 7.** Survival curves of the original mouse L·P3 cells and L·P3 Co-3 cells irradiated with various doses of X-rays. The survival curves were obtained from the mean of five independent experiments. Vertical bars show standard deviations.
method following irradiation with various doses of X-rays. As shown in Figure 7, L-P3 Co-3 cells were more radioresistant than the original L-P3 cells. D_0 and D_4 values of L-P3 Co-3 cells were 1.6 and 1.8 times higher than those of the original L-P3 cells respectively. NPSH content in both L-P3 and L-P3 Co-3 cells were also assayed at various culture days. As can be seen in Figure 8, L-P3 Co-3 cells contained about a 1.7 times larger amount of NPSH than in the original L-P3 cells at the second day of culture. This high concentration of NPSH in radioresistant L-P3 Co-3 cells was maintained through the first 4 days of culture. Then it gradually decreased and eventually reached the same level as that in the original L-P3 cells at the 7th day of culture.

The correlation between radioresistance of the cells and high content of NPSH has also been found in the cells in vivo. By repeatedly X-irradiating the Ehrlich ascites tumor cells with 2000 R and by repeatedly transplanting single cell into mouse
peritoneal cavity, Révész et al.\textsuperscript{11} isolated the radioresistant clones which have the elevated levels of NPSH content, and they suggested that the intrinsic NPSH level may be a factor in determining the cellular radiosensitivity. A similar correlation between radiosensitivity of the cells and cellular content of NPSH was found in the various sublines of ascites ovarian rat tumor cells\textsuperscript{2,7} and in the cells of various organs obtained from two strains of mice having different sensitivities to X-rays.\textsuperscript{13} On the other hand, this correlation has also been found in various phases in the cell cycle of cultured cells. Recently, Ohara and Terasima\textsuperscript{18} have found that the content of NPSH in HeLa cells is closely related to the cyclic variation of X-ray survivals during the cell cycle. Furthermore, Sinclair\textsuperscript{23} has reported that the cyclic variation of X-ray survival during the cell cycle in Chinese hamster cells were modified by the treatment of the cells with cysteamine and suggested that variations in intracellular NPSH in various cell phases might explain the cyclic age response to X-rays in mammalian cells.

The relationships between biochemical and genetical properties examined in the original HeLa S3, SM-1a and RM-1b cells and their radiosensitivities are summarized in Table 3. The APSH and protein contents were not significantly different among three cell lines. The differences in modal chromosome numbers of these cell lines did not reflect the differences in their radiosensitivities. Only different levels of NPSH content in the original HeLa S3, SM-1a and RM-1b cells were highly correlated to their radiosensitivities. It can be concluded, therefore, that the content of NPSH in the cells is the main factor in determining cellular radiosensitivity.

**Table 3**

| Cells   | NPSH content\textsuperscript{a} (×10\textsuperscript{-16} moles/cell) | APSH content\textsuperscript{b} (×10\textsuperscript{-14} moles/cell) | Protein content\textsuperscript{c} (µg/cell) | Modal chromosome number | Radiosensitivity D\textsubscript{0} (R) | D\textsubscript{2} (R) |
|---------|-------------------------------------------------|-------------------------------------------------|---------------------------------------|------------------------|--------------------------------------|----------------|
| HeLa S3 | 5.70 ± 0.39\textsuperscript{c}                  | 5.21                                            | 406.4 ± 45.0\textsuperscript{c}        | 68                     | 160                                  | 160 |
| SM-1a   | 5.02 ± 0.23                                     | 5.01                                            | 380.2 ± 30.2                         | 66                     | 110                                  | 140 |
| RM-1b   | 7.66 ± 0.41                                     | 5.03                                            | 375.6 ± 38.0                         | 62                     | 180                                  | 230 |

\textsuperscript{a} These values were the mean of five independent determinations.

\textsuperscript{b} These values were the mean of three independent determinations.

\textsuperscript{c} Standard deviation.

ACKNOWLEDGMENTS

The authors wish to thank Prof. H. Katsuta, The Institute of Medical Science, University of Tokyo, for his kind gift of mouse L·P3 and L·P3 Co-3 cells and Prof. T. Sugahara, Department of Experimental Radiology, Faculty of Medicine, Kyoto University, for his valuable advice and suggestions concerning this manuscript. This study was supported in part by Grant-in Aid for Cancer Research from The Ministry of Education, Science and Culture, Japan.
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