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MICROELECTROPHORETIC STUDY OF RADIATION-INDUCED DNA DAMAGES IN INDIVIDUAL MAMMALIAN CELLS

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SUMMARY: Mammalian cells were after irradiation suspended in melted agarose, and casted on microscope slides. The slides were after gelling at 0°C immersed in a neutral detergent solution which lysed the cells. A weak electric field (5 V/cm) was then applied over the gel for 5 minutes. The DNA in the gel was stained with the fluorescent dye acridine orange and gives a green emission in a microscope photometer. DNA had migrated towards the anode and this migration was more pronounced in irradiated than in control cells. The differences in migration pattern were quantitatively measured. The lower detection limit was below 0.5 Gy and a plateau in the dose-effect curve was reached at about 3 Gy. In repair experiments residual DNA damage could be observed after postirradiation incubation for 60 minutes.

The advantages of the method is: no radioactive labelling and only a few number of cells is required.

INTRODUCTION: Methods used for DNA strand break analysis often use a suspension of radioactively labelled cells. Most of these methods are now well established in several laboratories and refined in sensitivity, as for example ultracentrifugation in neutral (1) or alkaline (2) sucrose gradient, methods based on DNA unwinding in weak alkali (3), neutral (4) or alkaline (5) filter elution, nucleoid sedimentation (6) and viscoelastic measurements of DNA (7). One method developed previously in our laboratory is based on fluorescence measurements on single cells embedded in agarose on a glass slide after DNA unwinding in weak alkali and staining with AO[1] (8). In another method, presented in this paper, cells are similarly embedded in agarose on a glass slide, but instead of DNA unwinding, cells are lysed in a neutral detergent solu-

[1]Abbreviations used: AO = acridine orange, PBS = phosphate buffered salt solution, SDS = sodium dodecyl sulphate.

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tion and a weak electric field is then applied followed by staining with acridine orange and evaluation in a microscope photometer. These two methods have a common feature: a small number of cells are required and no radioactive labelling is needed. Due to the high sensitivity the method presented here has a high potential as a predictive test of the efficiency of radio- and chemotherapy of human tumours.

MATERIALS AND METHODS: A murine lymphoma cell line L5178Y-S was grown in suspension in Fisher's medium (GIBCO, U.S.A.) supplemented with 8 % donor calf serum (Flow Lab., U.K.) and antibiotics. Chinese hamster fibroblast cells (Cl-1) were grown as monolayers in plastic flasks (Nunc, Roskilde, Denmark) in Eagle's Minimum Essential Medium with Earle's salts supplemented with 20 % newborn calf serum (Flow Lab., U.K.) and antibiotics. The cultures were placed in a humified atmosphere of 5 % CO_2 in air at 37°C.

Irradiation with ^{60}Co gamma rays was performed at 0°C at a doserate of 1.8 Gy/min. Fibroblasts were trypsinized and suspended in PBS[1] before irradiation.

The cell suspension was after irradiation kept at 0°C or incubated at 37°C (repair study). An aliquot (usually 100 µl) of the cell suspension was suspended in 1 ml melted 0.75 % low gelling temperature agarose (FMC Corporation, Marine Colloids Div., USA) in PBS at 33°C giving a final concentration of about 10^4 cells per ml. The mixture was casted on a microscope slide pretreated with agarose of low concentration (0.1 %) in order to improve the adhesion of the 0.75 % agarose to the slide. After gelformation at 0°C, the slides were treated for 1 hour in a lysing solution containing 2.5 % SDS[1], 0.025 M EDTA, pH adjusted to 9.5, well below the limit for DNA unwinding (4). An electric field of about 5 V/cm was applied for 5 minutes and the slides were then washed in distilled water for 1 hour followed by air drying. The thickness of the agarose layer was now very thin. DNA was after another short washing in distilled water stained by immersing the slide in an AO solution 2 µg/ml (Polyscience, Inc., U.S.A.) in PBS for about 10 minutes and a coverslip was mounted so that the specimen was kept in the same solution.

Fluorescence was measured in a Leitz MPV2 microscope photometer 40x objective using a Phloemopak filterblock H2 giving excitation at 390-490 nm. The emitted light from individual cells passed a selection filter (525 nm) and thus the green fluorescence from the complex AO bound to double-stranded DNA was measured (AO bound to RNA or single-stranded DNA gives red fluorescence). Staining and fluorescence measurements were done within the first day after the experiment.

L5178Y-S cells were incubated for 24 hours in medium containing 0.1µCi/ml of [^{14}C]thymidine (Amersham International, U.K.) at 37°C. The cells were washed free from excess radioactivity and treated as above. The slides were after drying coated with emulsion (Ilford K5) by the dipping method and exposed for 8 weeks in dark. The slides were then developed (Kodak D-19 B), fixed, washed and mounted.

RESULTS: When embedded, the cell forms a cavity in the gel. After lysis of the cell its DNA occupies this cavity. Most other biomolecules in the cell are

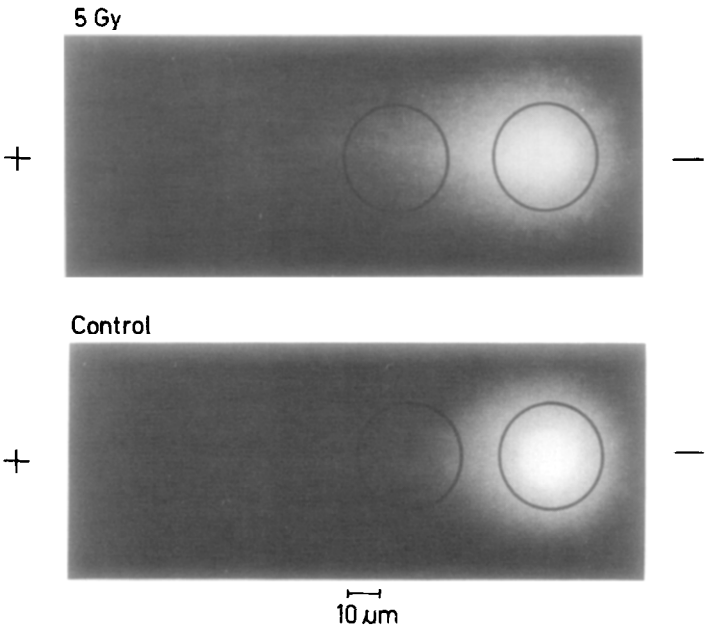

Fig. 1 Green fluorescence (525 nm) of DNA from an irradiated and control Cl-1 cell after electrophoresis.

readily diffusible through the agarose gel and thus there will be an almost total diffusion of these molecules out from the cavity into the lysing solution, except for the DNA with its extremely high molecular weight. During electrophoresis DNA will be partly liberated from the cavity and migrate towards the anode which is shown in Fig. 1 by fluorescence from DNA stained with AO and in Fig. 2 by ^{14}C-labelled DNA visualized by autoradiography. We call the DNA, which has migrated, for the "tail" and the remainder in the cavity for the "center". A fibrous structure can be seen at the end of the tail in high magnification (100x objective).

As can be seen in Fig. 1, more DNA was liberated from irradiated cells than from control cells. In order to quantify the liberation of DNA from the center, the green fluorescence was measured using a circular diaphragm first over the center and then at various positions of the tail (Fig. 1). For each cell the fluorescence background was measured beside the cell and subtracted from the measuring values. The time of illumination and between measurements were standardized in order to obtain similar fading in a measuring sequence.

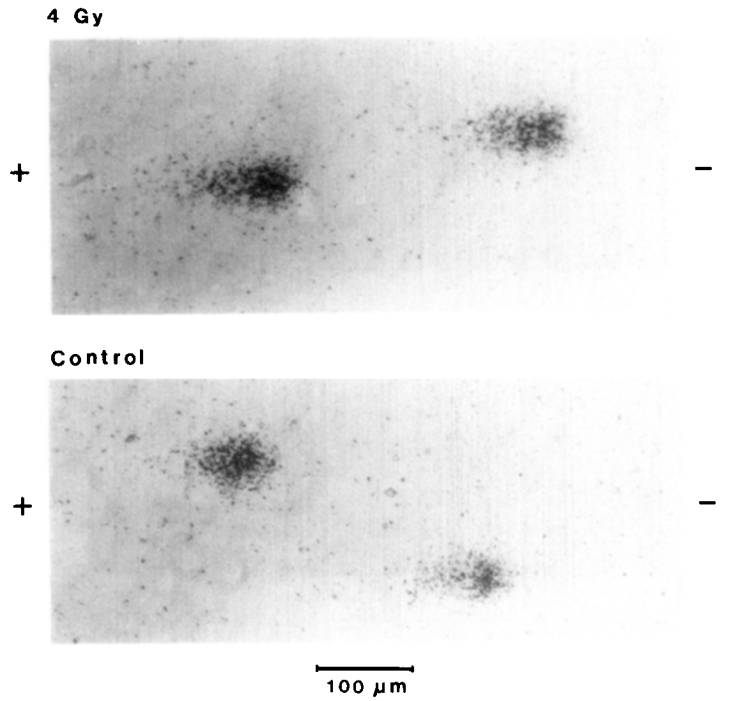

Fig. 2 Autoradiogram after electrophoresis of DNA from irradiated and control L5178Y-S cells which have incorporated [^{14}C]thymidine into DNA.

The results are in the following presented as the ratio of fluorescence (F_x) at x µm distance on the tail versus fluorescence at the center of the cavity (F_o).

The setting of x should be chosen to optimize the difference between the ratio F_x/F_o from control and irradiated cells. As can be seen in Fig. 3 the choice of x is not critical within the interval 25-75 µm, but of course the accuracy of x is of great importance when measuring. In Fig. 4 this is also illustrated with a greater variety of doses. Based on the results in Figs. 3 and 4 and on data not presented here, we conclude that 50 µm will give the best resolution of the method in the dose-ranges we mostly apply. A detailed study of the dose-effect relationship in the low dose-range using L5178Y-S cells is presented in Fig. 5. It can easily be seen that a plateau level is reached at about 3 Gy.

The repair kinetics of L5178Y-S cells irradiated with 2 Gy and incubated at 37°C is shown in Fig. 6. The kinetics is similar as found when DNA strand

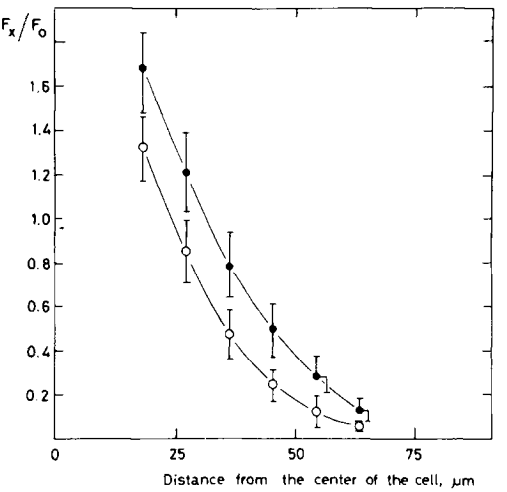

Fig. 3 The ratio of the fluorescence at various positions in the direction of migration versus the center of the cell (Cl-1). Filled symbols from cells irradiated with 7.5 Gy, and open symbols from control cells. Bars: S.D.

breaks are analysed with conventional methods. After incubation in 20 minutes we found a slight indication of newly formed breaks, recently proposed by Bryant et al. (9) as arosen by endonucleolytic incisions at base-damaged sites. After 2 Gy we are able to detect residual effects after 60 minutes incubation, independent of the cell-line we have used: cultured murine lymphoma cells, Chinese hamster fibroblasts or cells from irradiated human tumours *in vivo* from several experiments (results to be published). As can be seen in Fig. 7 these residual effects found 60 minutes postirradiation reach a plateau between about 10 and 30 Gy.

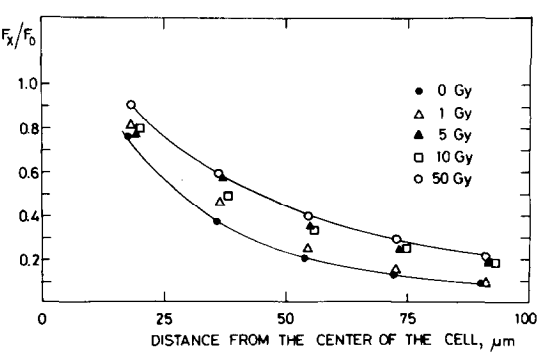

Fig. 4 The ratio of the fluorescence at various positions in the direction of migration versus the center of the cell (Cl-1).

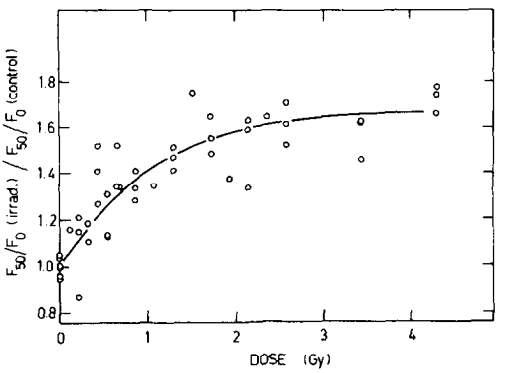

Fig. 5 A dose-effect curve for L5178Y-S cells. Fluorescence at 50 μm in the direction of migration versus the center of the cell. For every dose the ratio is normalized to the ratio from control cells. Each circle is the average from ten cells on one slide.

Several experiments have been done in order to optimize this method and these results will not be entirely presented here. A minor variation of (i) the concentration of cells on the slide, (ii) time of lysis, (iii) time of rinsing after electrophoresis, (iv) time of staining in AO, (v) concentration of AO, (vi) size of diaphragm in the microscope and, perhaps most interesting, (vii) time of electrophoresis were found to only slightly affect the results.

Contrary, a higher agarose concentration was obstructive for the migration and in some experiments we have observed that a delay between drying and staining in AO before evaluation could have some diminishing effect on the ratio F_X/F_0, i.e. a small contraction of DNA after micromigration can not be excluded. When waiting overnight for evaluation after staining the fluorescence had a tendency to fade. Most interesting for the discussion is that the

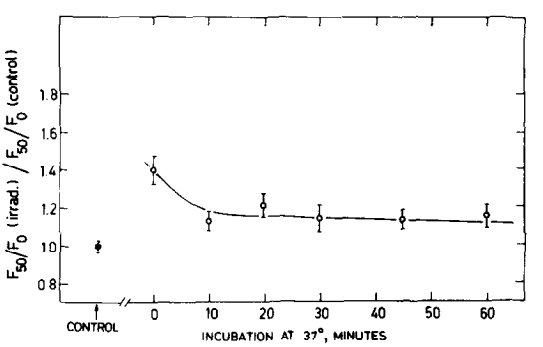

Fig. 6 Repair kinetics after irradiation with 2 Gy (L5178Y-S cells). Data presented as in Fig. 5. Bars: S.E.M.

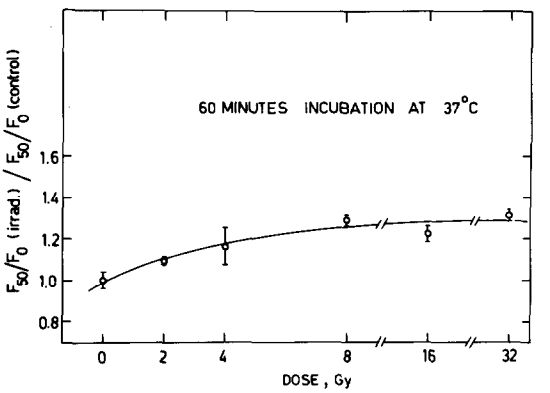

Fig. 7 Repair study on L5178Y-S cells when incubated from 60 minutes at 37°C after irradiation with different doses. Data presented as in Fig. 6. Bars: S.E.M.

strength of the electric field is important. For example, when applying 1 V/cm (instead of 5 V/cm) we were not able to observe any migration of DNA at all.

DISCUSSION: Since the cell and its DNA is treated very gently during embedding and lysis we expect that the molecular weight of DNA is very high and not far from that of DNA in living cells. It is certainly many magnitudes higher than the molecular weight of DNA used in conventional electrophoretic separations and any comparison with separation of DNA of 10^7 Daltons or less is not relevant.

The underlying mechanisms for the differences in the migration patterns observed in the present work is largely unknown. Our model for explanation of the phenomena is: DNA has an extremely high molecular weight and is organized similar as in the living cell, thus clusters of replicon still exist after lysis. When applying the electric field DNA is streched out toward the anode but will still be fixed in the cavity. DNA strand breaks introduce a relaxation of the super-coiling of DNA which results in a more pronounced migration of DNA towards the anode. The plateau level found at about 3 Gy (Fig. 5) should according to this explanation be a result of maximum relaxation of DNA supercoiling. There seems to be some similarities between our method and the nucleoid sedimentation method. Our model is merely a suggestion to explanation and other interpretations can not be excluded.

The repair kinetics indicates a fast process where more than 50 % of the introduced damages are repaired. This process might be rejoining of DNA single-strand breaks. After that period there is a rather slow repair phase and this might be repair of DNA double-strand breaks. If so, the method can be used to study the induction and repair of double-strand breaks after 2 Gy. The control value of the ratio F_{50}/F_0 varies with different cell populations with, e.g. variations of the size of the cell cavity and the amount of DNA, so we are not able to define a "standard control cell". It is thus important to have controls in each experiment and relate the migration of damaged cells to that of control cells. The low detection limit of the method requires cells in good condition. Other error sources of more or less importance are the quality of the glass slides (the heterogenic affinity of AO to these), the different fadings and the accuracy of x.

Compared to other methods the present method has some advantages, such as no radioactive labelling and only a few number of cells is needed which has enabled us to successfully work with cells from irradiated human tumours in vivo (results to be published).

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