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DNA LIGASE ACTIVITY IN CRUDE EXTRACTS OF FIBROBLASTS AND LYMPHOCYTES

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DNA ligase activity was determined in crude cell extracts using a new assay which measures the retention of double stranded circular phage λ DNA on nitrocellulose filters, and allows accurate determinations of the enzyme activity with cell concentration corresponding to 0.1 µg of proteins. Using this assay, we show that the DNA ligase activity varies greatly among mammalian cell lines. The higher activity is found in actively growing fibroblasts where it is stimulated by dimethyl sulfate pretreatment of the cells, whereas the low activity measured in resting lymphocytes is not modified by dimethyl sulfate. The DNA ligase activity correlates with the cells sensitivity towards ionizing radiations.

Mammalian cell lines differ widely in their capacity to repair DNA damage. For instance resting lymphocytes are one of the most sensitive cells to ionizing radiations. This radiosensitivity has been observed in vivo during cancer therapy (1), as well as in vitro experiments (2). Measurement of the repair of X-rays induced single strand breaks in the DNA of resting lymphocytes shows that DNA breaks persist after irradiation (3). This observation suggests that the lymphocytes radiosensitivity is related to a limited repair capacity towards these lesions.

Different enzymatic mechanisms are involveld in the repair of DNA (4). Among them, DNA ligase has been shown to repair DNA single-strand breaks, although this enzyme acts also during DNA replication (5). As a low level of DNA ligase could explain the persistance of DNA breaks observed in X-irradiated resting lymphocytes, we developped a simple and rapid assay to determine the ligase activity in cell extracts. This

Abbreviations used: PHA, phytohemagglutinin; DMS, dimethyl sulfate; DTT,dithiothreitol.

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assay is based on the property of double stranded circular phage λ DNA to be retained on nitrocellulose filters (6). That property was previously used to measure the activity of purified enzymes (7).

In this paper, we describe conditions which allow to measure the ligase activity in cell crude extracts, with a procedure with is fast, sensitive and quantitative. Using this assay, we show that the ligase activity is much more lower in resting lymphocytes than in actively growing cells.

Materials and Methods

Materials : The nitrocellulose filters (25 mm of diameter and 0.45 µm pore) were obtained from Schleicher & Schüll. Leupeptine and antipaine were from Peptide Institute Inc., Tokyo, Japan. Aprotinin was obtained from Laboratoires Choay, Paris, France.

Phage λ [^{14}C]DNA was prepared by thermal induction of E.coli 159 T$^-$ (λ cIts857 S7). Thymine [2-^{14}C] (specific activity : 19 mCi/mmole) was added immediately after induction. Bacteriophages were purified as described (8). After phenol extraction and dialysis against 2 M NaCl-10 mM Na_3-EDTA, the [^{14}C] labelled DNA was disaggregated by heating at 75°C for 5 min., and cyclised at 50°C for 2 hr (8). Its specific activity is 4.5 mCi/mmole (nucleotides).

Cell cultures: Chinese Hamster Ovary (CHO) cells were grown in Dulbecco's medium supplemented with 5 % fetal calf serum and 5 % horse serum in a 5 % CO_2 humidified atmosphere. The doubling time was about 14 hr. Transformed lymphocytes, strain B Priess (transformed by Epstein Barr virus), were obtained from Dr T. Tursz (Institut Gustave-Roussy). They were grown in suspension in RPMI 1640 medium supplemented with 20 % fetal calf serum, 200 mM glutamine, 30 % glucose and 100 mM Na pyruvate. Their doubling time was about 24 hours.

Lymphocytes : Human peripheral blood lymphocytes were isolated from heparinized venous blood of healthy donors by centrifugation on a Ficoll-Paque (Pharmacia) gradient (9). Purity of the preparations was about 85 %. For stimulation, lymphocytes were suspended ($1X10^6$cells/ml) in RPMI 1640 medium supplemented with 20 % fetal calf serum, 2 mM glutamine and PHA (25 µl/ml of cell suspension). They were maintained at 37°C in a 5 % CO_2 humidified atmosphere. Stimulation was checked by incubating the lymphocytes with [^{3}H] thymidine (2 µCi /culture) for 2 hours and measuring the amount of incorporated radioactivity.

Determination of ligase activity : Cells were suspended($2.5X10^6$cells/ml) in a buffer containing 20 mM Tris,HCl, 500 mM KCl, 2 mM DTT, pH 7.5. After sonication at 0°C (3 times, 15 sec), Triton X 100 (0,5 % final concentration) and 10 µg/ml of antipaine, leupeptine and aprotinin were added. Ligase reaction was carried out at 37°C in a medium containing 50 mM KCl - 10 mM $MgCl_2$ - 1 mM Na_3-EDTA - 50 mM Tris, HCl pH 7.5 -

100 µg/ml bovine serum albumine - 10 mM DTT - 0.16 mM ATP -40 µg/ml of each protease inhibitor - 1 µM (nucleotides) cyclised λ DNA and the appropriate quantity of cellular extract. The reaction was stopped by adding an equal volume of 4 M NaCl - 20mM Na_3-EDTA, and the mixture was heated at 75°C for 5 min., then quickly cooled at 0°C. After addition of 4.5 ml of 2.0 M KCl - 10 mM Na_3-EDTA, the samples were filtered through nitrocellulose membranes (previously soaked in water for several hours) at a flow rate of 1.5 ml/cm^2/min. The filters were rinsed with 1 ml of 0.01 N HCl, then dried and the radioactivity determined by liquid scintillation counting. Protein concentrations were determined according to Lowry (10).

Results and Discussion

A crude extract prepared from CHO cells is able to convert λ[^{14}C] DNA into a form which is retained by nitrocellulose filters in a time dependent reaction as shown in figure 1. Since in this assay, the substrate concentration ($S \simeq 2 \times 10^{-11}$M) is very low relatively to the Michaelis Constant (K_m) (5), the reaction rate is given by the equation :

$$S/S_o = \exp\left(-\frac{Vmax}{K_m}\, t\right) \qquad (1)$$

If log S is plotted as a function of time, a linear decrease is observed as expected (not shown). The quantity V_{max}/K_m appearing in

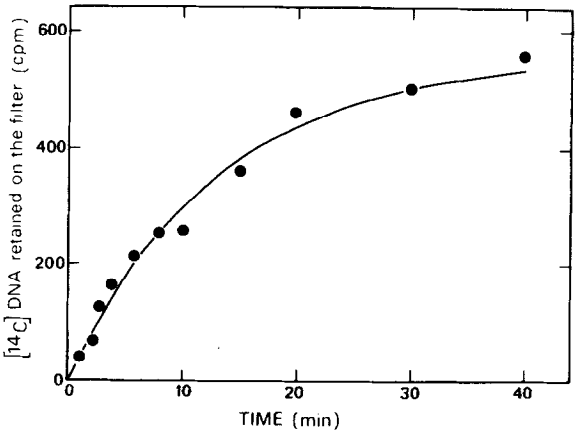

Figure 1:Kinetics of closure of λ DNA by a crude extract from CHO cells. The incubation was carried out at 37°C in the presence of a CHO cells crude extract (6.0 µg of proteins per ml corresponding to 7.7×10^{-2} units of activity). Aliquots of 0.1 ml were withdrawn at the indicated time and processed as described in Material and Methods. A background of 90 cpm determined at zero time was substracted from the countings.

equation (1) is proportional to the enzyme concentration. One can therefore define one unit of activity as the quantity of enzyme which catalyses the closure reaction with a rate constant of $V_{max}/K_m = 1\ min^{-1}$ in 1 ml of incubation mixture. When the filter assay is carried out at various protein concentrations (figure 2), the amount of $\lambda[^{14}C]$DNA retained by the filter after 15 min. of incubation increases linearly as a function of the protein concentration provided that less than about 25 % of the substrate is transformed. These results indicate that DNA ligase activity can be measured with accuracy and sensitivity in cell crude extracts. As little as 100 ng of proteins corresponding to about 1000 CHO cells is sufficient to cause the retention of a measurable amount of $\lambda[^{14}C]$DNA in the conditions of the assay.

Using this assay, the ligase activity was determined in different mammalian cell strains. The results, expressed in units of enzyme activity, were calculated from the linear part of the curves and are summarized in Table 1. Actively growing fibroblasts have the higher enzymatic activity which depends upon the presence of ATP in the incubation medium. The low enzymatic activity detected when ATP is omitted could be due to the small amount of this compound present in the cellular extracts (11).

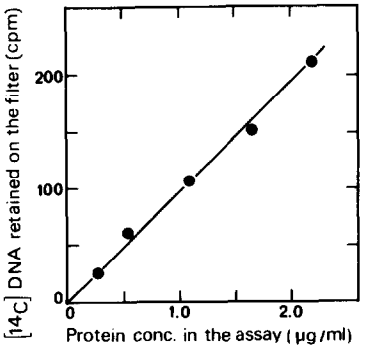

Figure 2:Relationship between the reaction rate and the protein concentration. Aliquots of 0.1 ml containing various amounts of CHO crude extract were incubated for 15 minutes and the quantity of transformed $\lambda[^{14}C]$DNA was measured by filter assay. A background of 122 cpm obtained in the absence of protein was substracted from the data.

Table 1. DNA ligase activity in crude extracts of different cells lines.

Cells	DNA-ligase Activity (Units / mg protein)
CHO cells	13.6
CHO cells (without ATP)	4.0
DMS-treated CHO cells	37.1
Transformed B lymphocytes	4.0
Transformed B lymphocytes (without ATP)	1.25
PHA-stimulated lymphocytes	1.22
Resting lymphocytes	0.12
DMS-treated resting lymphocytes	0.17

The enzymatic activity was determined as described in Materials and Methods in the presence of ATP or not. The lymphocytes were stimulated with PHA (25 µl/ml) for 72 hours. DMS-treated CHO cells or lymphocytes were incubated with 100 µM DMS for 30 minutes, and the enzymatic activity was measured 2 hours later.

We have found a large variation of the DNA ligase activity among the cell lines tested. Resting lymphocytes have the lower enzymatic activity which increases during PHA-stimulation. An increase of the DNA ligase activity during stimulation was already reported by Pedrini et al. (12). It should be noted that the DNA ligase activity parallels the radiosensitivity of the different cell lines studied (3).

A significant increase of the DNA ligase activity is measured when CHO cells are incubated with DMS. This result is in agreement with those of Mezzina et al. (13) who found an enhanced activity in human fibroblasts treated with various carcinogens. However, no significant modification is measured when resting lymphocytes are treated with DMS.

These results show that the DNA ligase activity varies greatly among mammalian cell strains. The assay, which needs low protein concen-

trations, could be used to determine this enzymatic activity in various cell mutants (14,15) and could contribute to better understanding of the role of DNA ligase in DNA repair processes.

Acknowledgments

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