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DETECTION OF DNA SINGLE-STRAND BREAKS AND UNSCHEDULED DNA SYNTHESIS INDUCED BY PROCARCINOGENS IN PRIMARY CULTURES OF RAT HEPATOCYTES*

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ABSTRACT: *Procarcinogen induced DNA single-strand breaks and unscheduled DNA synthesis were measured in primary rat hepatocytes culture. For DNA single-strand breaks assay, rat liver DNA was prelabeled by injecting ^{3}H-thymidine during the peak of DNA synthesis following partial hepatectomy. Hepatocytes were isolated from the rat 2 weeks after surgery by a collagenase perfusion technique and maintained as monolayers in serum free medium on collagen-coated culture dishes. DNA single-strand breaks were measured by the alkaline elution technique. Incorporation of ^{3}H-thymidine to hepatocytes DNA in the presence of hydroxyurea was used to measure unscheduled DNA synthesis. Hepatocellular DNA was isolated by retention on polyvinyl chloride filters. Aflatoxin B_1(AFB_1), 2-acetylaminofluorene (AAF), 2-aminofluorene (AF), and diethylnitrosamine (DEN) showed a dose-dependent response in both assays. DNA single-strand breaks assay was appear to be more sensitive than unscheduled DNA synthesis assay.*

Keywords: *DNA single-strand breaks, Unscheduled DNA synthesis, Procarcinogens, Hepatocytes culture.*

INTRODUCTION

Primary culture of rat hepatocytes has been widely used as an *in vitro* screening system for detecting carcinogenic chemicals since hepatocytes not only contain sufficient level of enzymes necessary for activation of the carcinogen to a chemically reactive form but also serve themselves as the target of the reaction. The measurement of chemically induced DNA repair as unscheduled DNA synthesis (UDS) and more recently determination of DNA single-strand breaks using alkaline elution technique have been extensively studied (Nicolini *et al.*, 1982; Williams, 1982). In previous reports we studied DNA single-strand breaks induced by procarcinogen in Chinese hamster ovary (CHO) cells cocultured with rat hepatocytes and carcinogen-induced UDS at different hepatocytes culture conditions and treatment times (Yang *et al.*, 1984; Kim *et al.*, 1985). The present study was designed to determine the sensitivity of UDS assay and DNA single-strand breaks assay in rat hepatocytes culture. For DNA single-strand breaks assay, hepatocellular DNA was prelabeled *in*

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vivo by injecting ^{3}H–thymidine after partial hepatectomy and hepatocyte cultures were made from the partial hepatectomized rat. For UDS assay, hepatocytes cultures were made from untreated rats and ^{3}H–thymidine added to the culture along with chemical.

MATERIALS AND METHODS

Primary Rat Hepatocytes Cultures

For DNA single–strand breaks assay, male Sprague–Dawley rats (100–120g) were patial hepatectomized according to the method of Higgins and Anerson (1931) and hepatocellular DNA was labeled by injecting ^{3}H–thymidine during the peak of DNA synthesis following the patial hepatectomy. ^{3}H–Thymidine was given ip at a dose of 50 μCi to animal every 4 hr beginning at 16 to 17 hr after the operation until each animal received a total of 400 μCi. The animal was used at which time the liver returned to a quiescent state. For UDS assay, rat (200–250g) was used without partial hepatectomy. Hepatocytes were isolated by a collagenase perfusion technique as reported previously (Yang *et al.*, 1983). Modified Waymonth's medium supplemented with hormones as described by Salocks *et al.* (1981) was used. The hepatocyte suspension was diluted 1×10^{6} cells per ml of medium and 3 ml were pipetted into 60×15 mm plastic petri dishes which were precoated with rat tail collagen. The culture incubated at 37°C in a humidified 5% CO_2/95% air incubator for the time indicated. The medium was changed 3 hr after initial plating to remove unattached cells and cellular debris.

DNA Single–Strand Breaks Assay

At 6 hr after initial plating, the medium was replaced with fresh medium containing hydroxyurea (10 mM) and 1–β–D–arabinofuranosylcytosine (ara–C, 10 μM) and carcinogen. Aflatoxin B_1 (AFB_1), 2–acetylaminofluorene (AAF) and 2–aminofluorene (AF) were dissolved in dimethylsulfoxide (DMSO) and diethylnitrosamine (DEN) was diluted with sterile water. The final concentration of DMSO was not exceed 1% in medium. After incubation of 4 to 24 hr as indicated, cells were harvested and the alkaline elution assay was perfomed as described previously (Yang *et al.*, 1984).

Unscheduled DNA Synthesis Assay

The experimental protocol for UDS assay was similar to that of DNA single–strand breaks assay. Hepatocytes cultures made from untreated rat were incubated for 6 hr and the fresh medium containing hydroxyurea (10 mM), ^{3}H–thymidine (1 μCi/ml), and carcinogen was added. After incubation for the period specified, the medium was aspirated off and the plate was washed twice with phosphate–buffered saline (PBS) containing thymidine (0.5 mg/ml) and the hepatocytes were harvested.

The filter elution method used for the detection of DNA single–strand breaks was used to measure unscheduled DNA synthesis according to the modification of Hsia *et al.* (1983). The plate which was rinsed with PBS containing 0.5 mg/ml cold thymidine was treated with EDTA solution for 10 min at 37°C and the cells were detached by gentle scrapping with a rubber policeman and dispersed in cold PBS solution. About 6×10^{6} cells were loaded onto a 2.0 μm pore size polyvinylchloride filter in a 25 mm filter holder. The cells were lysed in the dark with 10 ml of lysis solution (2% sodium dodecylsulfate, 0.025M EDTA, 0.1 M glycine, pH 10.0) supplemented with thymidine (0.5 mg/ml) and proteinase K (0.2 mg/ml). The lysis solution allowed to flow by gravity. When 1 ml of the lysis solution remained on the filter, the outflow tube from the filter holder was clamped off and incubation was continued for 30 min at room teperature. After incubation, the samples were allowed to drip to dryness. The lysed samples were washed with 5 ml of 0.02M EDTA solution (pH 10.0). After final washing, each filter was carefully removed and placed in a scintillation vial to

which 0.4 ml of HCl was added. The vial was heated for an hour at 70°C. After cooling, 0.5 ml of 2N NaOH was added. The vials were placed in a shaker bath at 25°C and were shaken vigorously for 1 hr. Finally, 0.4 ml of aliquots was taken and 7 ml of Aquasol containing 0.7% acetic acid were then added to each vial for radioactivity counting. For DNA assay, 0.5 ml of aliquots was taken and DNA concentration was measured by the diphenylamine procedure of Burton (1956).

RESULTS AND DISCUSSION

The initial experiments determined DNA single–strand breaks induced by AFB_1, (5×10^{-6} M) at different hepatocytes culture ages. As shown in Table 1, DNA single–strand breaks induced by AFB_1 was maximum at earlier culture age (6 hr) and decreased considerably thereafter. In hepatocyte cultures, it has been demonstrated that the level of cytochrome P–450, the terminal oxidase responsible for the metabolism of many drugs, declines rapidly with culture time (Michalopoulos *et al.*, 1976). Therefore, the decreased response of hepatocytes culture with age might due to decreased metabolic capabilities of hepatocytes. As the results of this experiment, 6 hr hepatocytes cultures were used in the remaining DNA single–strand breaks and UDS assays.

Table 1. DNA single–strand breaks induced by aflatoxinin B_1 in primary rat hepatocytes culture at different culture ages[a].

Culture Age (hr)	Increased Elution (%) (Durg–Control)
6 hr	37.9±2.8[b]
12 hr	34.0±3.9
24 hr	30.6±3.1

[a]The hepatocytes were exposed to 5×10^{-6} M of AFB_1 for 4 hr at indicated times after plating.
[b]Mean±S.E. for quadraplicate cultures.

In Fig. 1 hepatocytes cultures were treated with carcinogens for different time period and resulting DNA single–strand breaks were determined. DNN single–strand breaks induced by AFB_1, AAF and AF increased with longer treatment time. In case of DEN, maximum values were obtained at 12 hr exposure and did not increased at 24 hr treatment.

Fig. 2 shows DNA single–strand breaks and UDS induced by AFB_1. Results of both assays were quite comparable and good dose–related response was obtained. DNA single–strand breaks assay appears to be more sensitive than UDS assay by showing a significant difference at 1×10^{-8} M; a significant difference was observed at 5×10^{-8} M in UDS assay. The reduced UDS response observed at 1×10^{-5} M AFB_1 was attibuted to the cytotoxicity of aflatoxin.

Fig. 3 shows results of AAF treatment. AAF also induced a dose–dependent increases of DNA single–strand breaks and UDS in hepatocytes culture. The minimum detectable dose for DNA single–strand breaks and UDS were 5×10^{-7} and 1×10^{-6} M, respectiviely.

Fig. 4,5 shows DNA single–strand breaks and UDS induced by AF and DEN treatment, respectively. In case of AF, the minimum detectable dose for both assay was 1×10^{-7} M. In UDS assay, decreased response was observed at higher dose (1×10^{-5} M) due to the cytotxicity of AF. In case of DEN treatment, minimum detectable dose for DNA single–strand breaks was 5×10^{-4} M; while a significant difference was observed at 5×10^{-3} M for UDS assay.

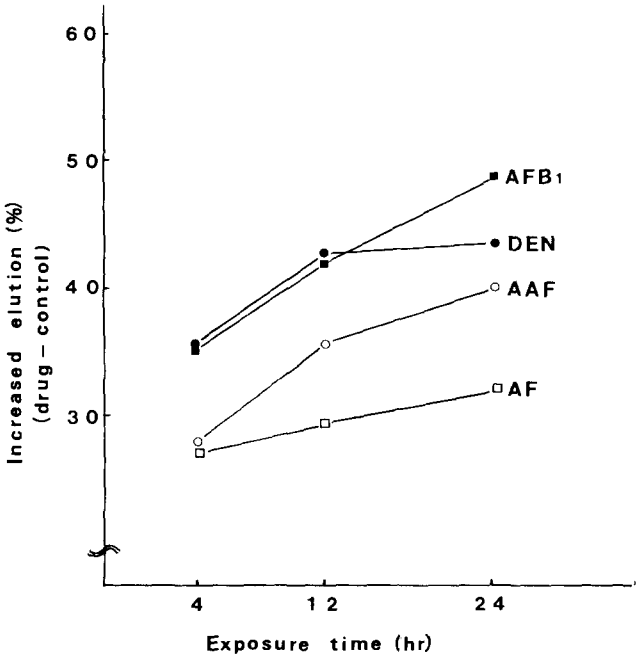

Fig. 1. Effect of exposure time on DNA single-strand breaks induced by procarcinogens. Hepatocytes were cultured for 6 hr before use. AFB_1 (5×10^{-6} M), AAF (1×10^{-5} M), AF (1×10^{-5} M), DEN (1×10^{-2} M). Each point represents the mean of duplicate determinations.

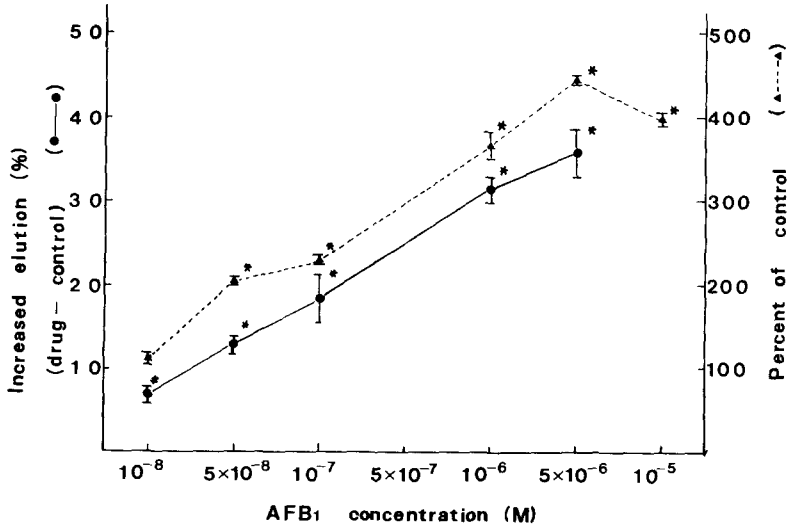

Fig. 2. DNA single-strand breaks (●——●) and UDS (▲·····▲) induced by AFB_1 in hepatocytes culture. Hepatocytes were cultured for 6 hr before use and AFB_1 was treated for 4 hr. DNA single-strand breaks were expressed as the increased elution over that observed in the controls and UDS was expressed as a percent of controls. Each point represents the mean ± SE of three determinations. An asterisk indicates values significantly different from the controls ($p<0.05$).

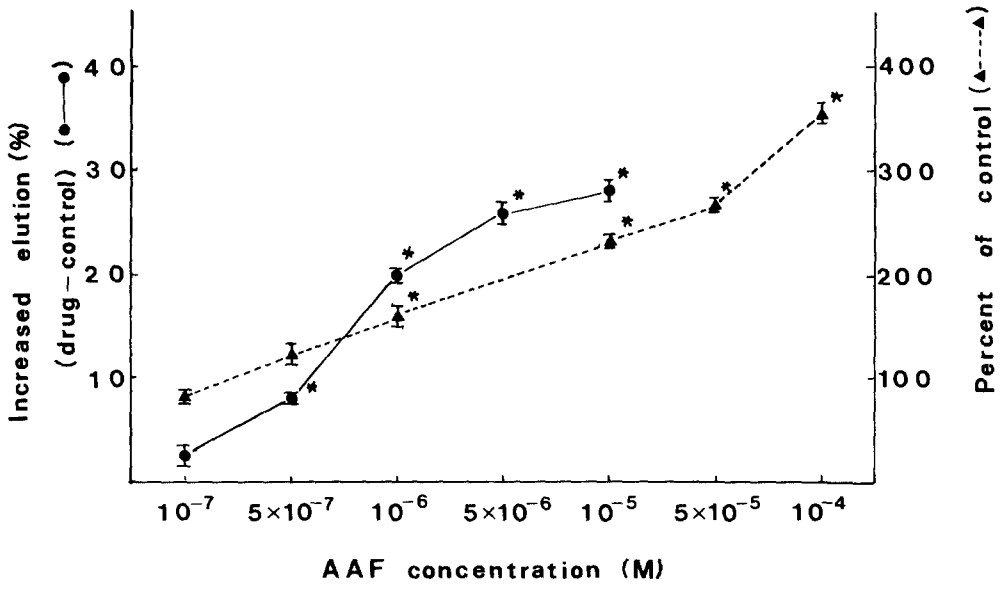

Fig. 3. DNA single-strand breaks (●——●) and UDS (▲·····▲) induced by AAF in hepatocytes culture. Experimental conditions are same as in Fig. 2.

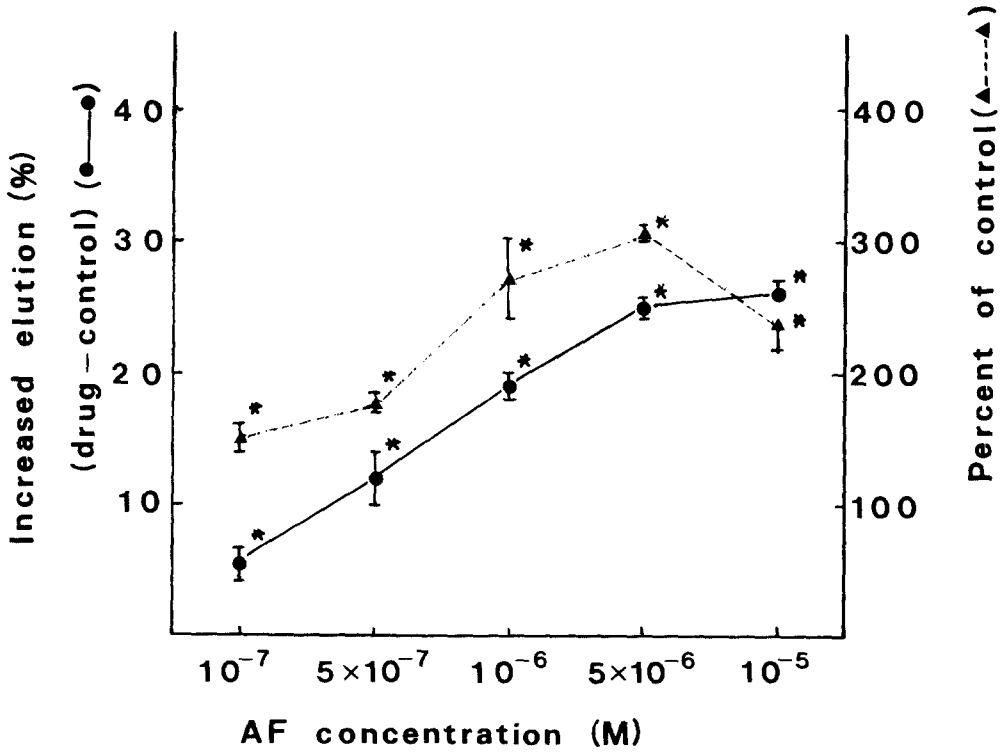

Fig. 4. DNA single-strand breaks (●——●) and UDS (▲·····▲) induced by AF in hepatocytes culture. Experimental conditions are same as in Fig. 2.

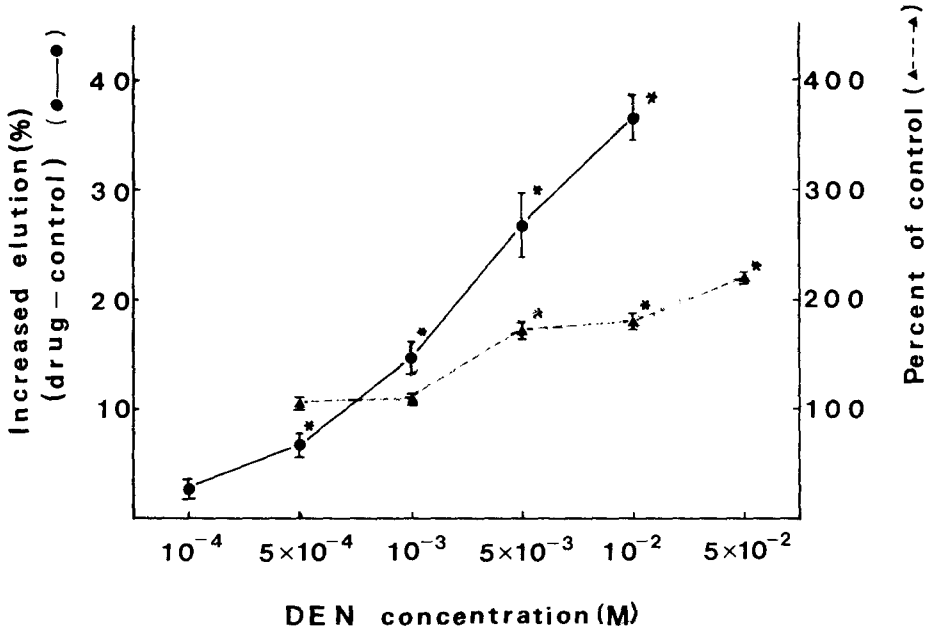

Fig. 5. DNA single–strand breaks (●——●) and UDS (▲·····▲) induced by DEN in hepatocytes culture. Experimental conditions are same as in Fig. 2.

In conclusion, AFB_1, AAF, AF and DEN induced a dose–related increases of DNA single–strand breaks and UDS in hepatocytes culture. Results of both assays were generally comparable, but DNA single strand breaks assay was appear to be more sensitive than UDS assay. Bradley *et al.* (1982) also reported that DNA single–strand breaks assay by alkaline elution technique was 500 to 1,000 times more sensitive than UDS for diethylnitrosamine. DNA single–strand breaks detected by alkaline elution may be derivated from several different mechanisms: by direct scission of the sugar phosphate backbone; by hydrolysis of alkali–labile sites; during base or nucleotide excision repair; during postreplication or recombination repair. The different sensitivity between two methods may be due to these reasons. In general, simultaneous determination of DNA repair and damage in a single population of cells not only provides information as to the kinetics of DNA damage but also measure the true genotoxicity of test compounds.

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