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# Simultaneous Measurement of Unscheduled and Replicating DNA Synthesis by Means of a New Cell Culture Insert DNA Retention Method: Rapid Induction of Replicating DNA Synthesis in Response to Genotoxic Carcinogens

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**In order to measure simultaneously replicating DNA synthesis (RDS) and unscheduled DNA synthesis (UDS) in rat hepatocytes responding to exposure to carcinogens, a new method, namely the "cell culture insert DNA retention (CDR)" method, was developed. All CDR procedures for cell culture, digestion of cytoplasm and retention of DNA were performed on membranes attached to cell culture containers. Four subgroups of primary cultures of hepatocytes prepared from rats were exposed to a genotoxic or non-genotoxic carcinogen with or without 10 m*M* hydroxyurea and incubated for 4 h with 10 $\mu$Ci/ml [$^3$H]thymidine. The membranes were then processed for both liquid scintillation and autoradiography. Among seven tested chemicals, three genotoxic agents, 3,2′-dimethyl-4-aminobiphenyl, 2-acetylaminofluorene and diethylnitrosamine, and two non-genotoxic carcinogens, nafenopin and phenobarbital, induced RDS within 4 h after the exposure, indicating that these carcinogenic agents induce cell proliferation in non-proliferating rat hepatocytes prior to the emergence of genotoxic changes. Several indices were devised to characterize the genotoxicity of the tested chemicals. The induction patterns obtained showed a wide variation in the individual characteristics of carcinogen-induced genotoxicity and mitogenicity in the early phase of initiation. This is the first report of simultaneous measurement, by using a combination of autoradiography and liquid scintillation, of UDS and RDS induced in rat hepatocytes. The described CDR approach will be useful for risk assessment and characterization of carcinogenic and tumor-promoting agents.**

Key words: Risk assessment — Characterization of genotoxicity and mitogenicity — UDS — RDS — Cell proliferation

There are numerous synthetic or naturally occurring carcinogens, including non-genotoxic types, in our environment.[1–4] Ubiquitous exposure to environmental mutagens and carcinogens is one of the major problems facing mankind,[5, 6] and screening and risk assessment of such chemicals are particularly important issues.[7, 8] *In vitro* short-term assays such as Ames' test and hepatocyte culture/DNA repair assay have been commonly employed for this purpose.[9–11] Optimally, cell culture systems for detecting agents with carcinogenic potential should be broadly sensitive to a variety of carcinogens, including procarcinogens that require metabolic activation. Among short-term tests, that using mammalian hepatocytes is regarded as reliable, since hepatocytes carry out numerous enzymatic reactions (allowing the detection of metabolic products) and are non-replicating for 48 h after collection.[12–16]

Each assay for testing of DNA damage and repair has certain advantages and disadvantages.[17] Hepatocyte culture/DNA repair assays are commonly employed for measurement of unscheduled DNA synthesis (UDS) using autoradiographic scoring of incorporated [$^3$H]-thymidine.[11, 18–29] However, the subtraction of cytoplasmic grain count, usually carried out to obtain the net nuclear grain count, may represent a potential source of error when the test compound is weakly genotoxic in the assay. Furthermore, UDS in S phase cells has sometimes been ignored in hepatocyte culture/DNA repair assay. Liquid scintillation is another method for detection of UDS,[18, 30–32] but its use is hampered by the difficulty in distinguishing UDS from replicating DNA synthesis (RDS), even when antimetabolites such as hydroxyurea (HU) or aphidicolin are applied. Nevertheless, the scintillation method for UDS is considered to be more accurate than autoradiography, if complete elimination of RDS is possible.

Recently, evidence suggesting that mitogenesis (induced cell division) plays a dominant role in carcinogenesis has been accumulated.[33–38] Chronic proliferation induced via compensatory mechanisms has been well analyzed, but the direct mitogenic action of chemicals in the early phase of initiation is still unclear. Accordingly, simultaneous analysis of genotoxicity and mitogenicity of chemicals at various doses should be an advantageous approach for studying mechanisms of action, in addition to simple screening of carcinogens. In the present report, a new, rapid *in vitro* system for this purpose and the induction of UDS and RDS by several genotoxic and non-genotoxic carcinogens are described.

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## MATERIALS AND METHODS

**Materials** A 25 mm cell culture insert container and membrane, Falcon 3091 (Becton Dickinson Labware, Lincoln Park, NJ) was employed for the cell culture, digestion of cytoplasm and retention of DNA. Cell culture and all incubations were performed on 6 well plates provided by Flow Laboratories Inc. (McLean, VA). Culture media does not pass through the pores of the membrane (3.0 $\mu$m in diameter) owing to surface tension. The liquid absorbing material (LAM, Kaoh, Tokyo) used was made up of a mixture of cotton and dry-gel particles and the surface was covered with soft paper. The undersurface and margins were covered with vinyl sheeting. All washes through the culture insert membrane were performed on LAM and the discharges were aspirated through capillary pressure into LAM. [Methyl-$^3$H]thymidine (1.5–2.2 TBq/m*M*) was purchased from Amersham Japan Inc. (Tokyo). Five genotoxic carcinogens, 2-acetylaminofluorene (2-AAF, Nacalai Tesque, Kyoto), aflatoxin $B_1$ ($AFB_1$, Makor Chemicals Co., Israel), 7,12-dimethylbenz[a]anthracene (DMBA, Nacalai Tesque, Kyoto), diethylnitrosamine (DEN, Nacalai Tesque) and 3,2′-dimethyl-4-aminobiphenyl (DMAB, Sigma Chemical Co., St. Louis, MO), as well as two non-genotoxic carcinogens, nafenopin (NF, Ciba-Geigy, Basel, Switzerland) and phenobarbital (PB, Maruishi Chemical Co., Ltd., Osaka), were used as representative test samples.

**Solutions** Williams' Medium E Incomplete (WEI): 500 ml of WEI was made by adding 50 mg of streptomycin (Meiji Seika Inc., Tokyo) and 0.15 g of L-glutamine (Nissui Pharmaceutical Co., Ltd., Tokyo) to Williams' Medium E (GIBCO/BRL Life Technologies, Inc., NY). Williams' Medium E Complete (WEC) contained 10% fetal bovine serum (FBS) (GIBCO/BRL Life Technologies, Inc.).

*Suppression medium*: WEI was used as the suppression medium for subgroups 1 and 3. WEI adjusted with 100 m*M* HU (Nacalai Tesque) was used as the suppression medium for subgroups 2 and 4 (final concentration during incubation was 10 m*M*).

*RI medium*: WEI containing 100 $\mu$Ci/ml [$^3$H]thymidine was prepared (final concentration during incubation was 10 $\mu$Ci/ml).

*Sample medium*: Chemicals were dissolved in dimethyl sulfoxide (DMSO, Sigma) or *N,N*-dimethylformamide (DMFA, Kishida Chem Co., Osaka) (final concentration of DMSO or DMFA during incubation was 0.1%). Sample medium of subgroups 3 and 4 contained appropriate concentrations of sample chemicals. WEI was used as the sample medium for subgroups 1 and 2.

**Protocol** Hepatocytes were isolated from nine- to eleven-week-old male ACI/N rats (200–220 g) purchased from Clea Japan., Inc. (Tokyo) and collected by a modification of the method described by Williams.[18] Perfusion of EGTA was terminated within 5 min and perfusion of collagenase I within 12 min to maintain high viability of cells. The isolated hepatocytes were suspended in 30 ml of WEC (3% FBS) and centrifuged at 500 rpm for 3 min using a Kubota KS-4000 rotor. The process was repeated until cell debris was completely removed. Finally, cell suspension was prepared at $2.0\times10^5$ viable cells/ml in WEC and 700 $\mu$l aliquots were distributed into the cell culture inserts (Fig. 1). Then 100 $\mu$l of suppression medium was added and the inserts were incubated for 1 h at 37°C under 100% humidity. Aliquots of RI medium (100 $\mu$l) and the same volume of sample medium were overlaid, and the incubation was continued for an additional 4 h. After the incubation, the following steps were performed at room temperature. The culture inserts were placed on LAM and the medium was aspirated off. Cells were washed twice with 1 ml of 1% sodium citrate. Hepatocytes were first swollen in 2 ml of 1% sodium citrate for 10 min, then incubated in 2 ml of 0.05% pepsin/0.2% HCl for 4 min. The cells were washed with four changes of 2 ml of 100% ethanol: glacial acetic acid (3 : 1) each for 20 min. The membranes were washed with 2 ml of 100% ethanol, dried and processed for liquid scintillation or autoradiography.

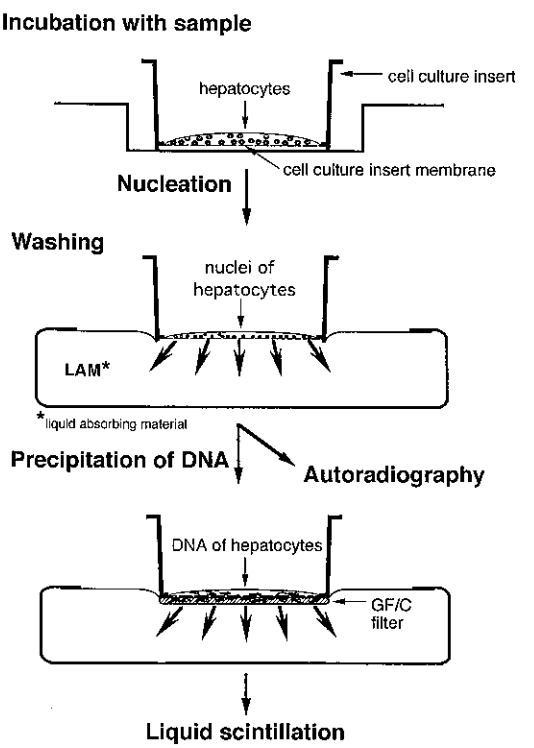


Fig. 1. The processes of incubation, nucleation and purification of DNA.

**Measurement of thymidine incorporation by liquid scintillation** Non-specific radioactivity was removed by a modification of the acid precipitation procedure described previously.[39-41] Briefly, fixed nuclei on the membrane were dissolved by incubation in 0.75 ml of 0.33 *N* NaOH for 30 min. DNA-protein complexes were precipitated by addition of 0.25 ml of ice-cold 40% TCA and 1.2 *N* HCl for 30 min. A GF/C filter 25 mm in diameter (Whatman International Ltd., England) was placed beneath the membrane, and the precipitate was washed with 2 ml of ice-cold 5% TCA, with 2 ml of 1 m*M* non-radioactive thymidine, twice with 2 ml of distilled water and three times with 2 ml of 100% ethanol on LAM. Both halves of cell culture insert membranes cut with a razor blade and the GF/C filters were placed in a 20 ml scintillation vial and moistened with a 150 $\mu$l aliquot of distilled water for 30 min. The precipitate was solubilized by incubation in 500 $\mu$l of Soluene-350 (Packard Japan, Tokyo) for 30 min, then 10 ml of scintillation cocktail, Hionic-Fluor (Packard), was poured in and the vials were allowed to stand for 30 min in darkness. Radioactivity of [$^3$H]thymidine incorporated into DNA was measured by a Minaxi-beta 4000 series liquid scintillation counter (Packard). Raw data were analyzed using the following calculation and values appearing in figures are the averages of the values in triplicate determinations along with the standard deviation.

**Calculation of ΔUDS and ΔRDS and indices for genotoxicity** Fig. 2 illustrates the effects of HU and the genotoxic agents (GA) on hepatocyte DNA synthesis. Total DNA synthesis (TDS) consists of RDS and UDS, and was measured by scintillation counting. The use of 0.1% DMSO as the solvent control induces $TDS_1$ (Fig. 2, subgroup 1, HU−, GA−), consisting of $RDS_1$ (=base line RDS) and $UDS_1$ (=base line UDS),

$$TDS_1 = RDS_1 + UDS_1 \quad [1]$$

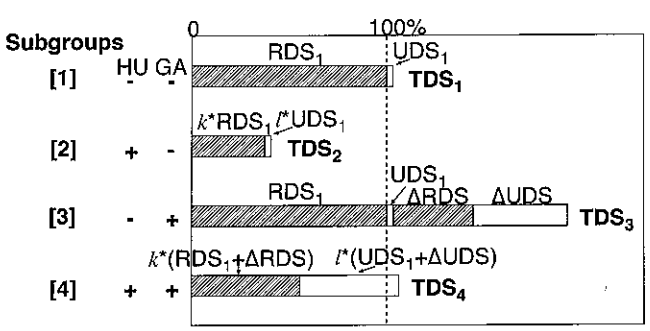


Fig. 2. Influence of HU and GA on hepatocyte DNA synthesis.

GAs induce ΔUDS and ΔRDS (subgroup 3, HU−, GA+),

$$TDS_3 = TDS_1 + \Delta RDS + \Delta UDS = (RDS_1 + UDS_1) + \Delta RDS + \Delta UDS \quad [2]$$

HU inhibits RDS by $k$ and influences UDS by $l$, thus reducing $TDS_1$ to $TDS_2$ (subgroup 2, HU+, GA−) and $TDS_3$ to $TDS_4$ (subgroup 4, HU+, GA+),

$$TDS_2 = k \times RDS_1 + l \times UDS_1 \quad [3]$$

$$TDS_4 = k \times (RDS_1 + \Delta RDS) + l \times (UDS_1 + \Delta UDS) \quad [4]$$

The inhibiting effect of HU is more specific for RDS than UDS ($0 < k \ll l$). The $k$ value is estimated by liquid scintillation and the $l$ value by the autoradiographical method described later. The solutions of the simultaneous equations [1]–[4] are,

$$RDS_1 = \frac{l \times TDS_1 - TDS_2}{l - k} \quad [5]$$

$$UDS_1 = \frac{TDS_2 - k \times TDS_1}{l - k} \quad [6]$$

$$\Delta RDS = \frac{l \times (TDS_3 - TDS_1) - (TDS_4 - TDS_2)}{l - k} \quad [7]$$

$$\Delta UDS = \frac{(TDS_4 - TDS_2) - k \times (TDS_3 - TDS_1)}{l - k} \quad [8]$$

$$\text{Total RDS} = RDS_1 + \Delta RDS = \frac{l \times TDS_3 - TDS_4}{l - k} \quad [9]$$

$$\text{Total UDS} = UDS_1 + \Delta UDS = \frac{TDS_4 - k \times TDS_3}{l - k} \quad [10]$$

FBS is an ideal sample for the estimation of $k$ value, which influences RDS, but never induces DNA damage (Fig. 2, $\Delta UDS = 0$, $\Delta RDS \neq 0$). The $k$ value can be estimated from the expression [8],

$$\Delta UDS = \frac{(TDS_4 - TDS_2) - k \times (TDS_3 - TDS_1)}{l - k} = 0$$

$$k = \frac{TDS_4 - TDS_2}{TDS_3 - TDS_1} \quad [11]$$

**Autoradiography** Pairs of dried culture insert membranes were placed on slide glasses with 2 drops of Bioleit (Kohken Co., Ltd., Tokyo). Then the slide glasses were coated with 1 : 1 water-diluted NR-M2 emulsion (Konica, Tokyo), stored in a dark box for 21 days, developed and fixed according to the manufacturer's instructions. Specimens were then stained with hematoxylin and eosin and were examined at×1000 magnification for counts of UDS grains and calculation of labeling indices. Fields were randomly selected, and all of the hepatocytes in each field assessed. This procedure was repeated until at least 300 cells had been counted. The replication labeling indices were expressed as mean percentages of nuclei that were heavily labeled with [$^3$H]-thymidine. Unless otherwise stated, data are expressed as

mean±SD derived from at least 3 independent experiments.

**Indices** Amounts of DNA synthesis induced by chemicals were expressed as percentages of $RDS_1$ values. The indices below can be generally used to describe genotoxicity and mitogenicity of chemicals because they are independent of the amount of DNA applied or the model of scintillation counter.

$$\text{Repair index} = \frac{\text{total UDS}}{RDS_1} \times 100 \quad (\%) \qquad [12]$$

$$\text{Proliferation index} = \frac{\text{total RDS}}{RDS_1} \times 100 \quad (\%) \qquad [13]$$

$$\text{Induction specificity} = \frac{\Delta RDS}{\Delta UDS} \quad (\%) \qquad [14]$$

$$\Delta RDS \text{ effect} = \frac{\Delta RDS}{RDS_1} \times \frac{\Delta UDS}{RDS_1} \times 100 \quad (\%) \qquad [15]$$

## RESULTS

**Cell culture** The viability of the cultured hepatocytes after incubation was always more than 90%. The medium did not passed through the culture insert membrane as long as its undersurface was kept dry because of surface tension. The aspiration of the medium into LAM was completed within a few seconds. Moisture around the cells was maintained even after aspiration and more than 99.998% of nuclei were confirmed to be intact in the culture insert under a phase contrast microscope (Fig. 3) (less than 10 cells out of $5\times10^5$ cells were lost).

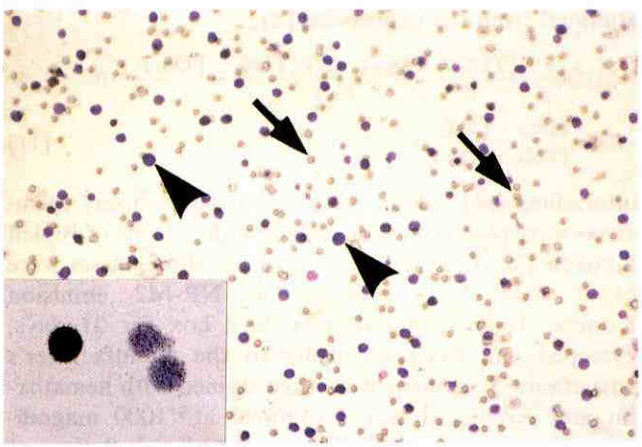

Fig. 3. Micrograph of processed membrane. Nuclei of hepatocytes (◄) and pores (►) of the cell culture insert membrane are visible (HE stain, original magnification ×210). The inserted micrograph shows a replicating nucleus (completely black) with incorporation of thymidine, and UDS on non-S phase nuclei (aflatoxin $B_1$, $10^{-5}$ *M*; original magnification × 850).

**Liquid scintillation** The cell culture insert membrane could be processed intact through a series of fixative solutions and scintillation fluids. Hepatocytes were effectively swollen by 1% sodium citrate, and connections of cytoplasmic components were loosened by pepsin. The cytoplasmic structures were completely dissolved by 100% ethanol : glacial acetic acid (3 : 1) and nuclei were fixed. TCA at 10% final concentration effectively precipitated DNA on the cell culture insert membranes and GF/C filters.

FBS added for estimation of the *k* value reduced RDS by approximately 30% (Table I). HU suppressed RDS by approximately 35%. The range of $RDS_1$ was 1971–5374 cpm and that of $UDS_1$ was −66–167 cpm (Table II).

Values for DNA synthesis induced by genotoxic and non-genotoxic agents were calculated (Table III). Total UDS and total RDS, expressed as indices, are illustrated in Fig. 4. Treatment with $AFB_1$, 2-AAF or DMAB was associated with a larger total amount of DNA synthesis than in the case of the other chemicals tested. DMAB induced a smaller level of UDS but a larger level of RDS at $10^{-5}$ *M* and $10^{-6}$ *M*. However, no induction of RDS was apparent at $10^{-4}$ *M*. $AFB_1$ induced the largest UDS values in a dose-dependent manner as well as causing mitoinhibition at $10^{-5}$ *M*, so that UDS was 4 times the residual RDS. DEN at $10^{-5}$–$10^{-7}$ *M* induced smaller levels of UDS than the other genotoxic carcinogens and no induction was apparent at the $10^{-4}$ *M* level, despite an increase in RDS. Of interest was the finding that 2-AAF elevated RDS as well as UDS. In contrast, NF and PB increased only RDS.

**Autoradiography** The membranes could be easily cut with a razor blade and proved stable under the moist processing conditions used for mounting on slide glasses. Cytoplasmic structures including mitochondria and glycogen particles were completely removed and nuclei were confirmed to be intact. There were no significant differences in UDS grains between subgroups with HU (subgroups 1 and 3) and those without HU (subgroups 2 and 4). As a result, the *l* value was estimated at 1.0.

## DISCUSSION

The DNA extraction and alkaline elution methods which have been generally employed for liquid scintillation analysis of chemically induced DNA synthesis are complex and require numerous procedures, i.e., cell culture, scraping of cells, removal of cytoplasmic structures, purification of DNA and corrections based on DNA concentration. Also, these processes are relatively time-consuming and generate large volumes of radioactive effluent.

Rapid methods to identify genotoxicity and carcinogenicity of chemicals should preferably be: 1) broadly

Table I. Raw Data (cpm)

| Samples | $TDS_1$ | $TDS_2$ | $TDS_3$ | $TDS_4$ |
|---|---|---|---|---|
| $AFB_1$ control | 2102±58 | 855±23 | | $k$=0.425 |
| FBS | | | 1406±7 | 561±17 |
| $10^{-5}$ $M$ | | | 3942±59 | 3396±81 |
| $10^{-6}$ $M$ | | | 3145±112 | 2698±31 |
| $10^{-7}$ $M$ | | | 2748±40 | 2063±60 |
| $10^{-8}$ $M$ | | | 2662±51 | 1395±16 |
| DMBA control | 5394±121 | 1097±15 | | $k$=0.200 |
| FBS | | | 3577±329 | 732±35 |
| $10^{-4}$ $M$ | | | 5590±74 | 2215±67 |
| $10^{-5}$ $M$ | | | 4825±46 | 1445±38 |
| $10^{-6}$ $M$ | | | 5218±39 | 1132±24 |
| $10^{-7}$ $M$ | | | 5744±58 | 1130±50 |
| DEN control | 2687±102 | 681±27 | | $k$=0.238 |
| FBS | | | 1875±162 | 490±39 |
| $10^{-4}$ $M$ | | | 3041±148 | 730±96 |
| $10^{-5}$ $M$ | | | 2849±127 | 763±48 |
| $10^{-6}$ $M$ | | | 2978±215 | 928±36 |
| $10^{-7}$ $M$ | | | 3035±91 | 807±36 |
| 2-AAF control | 3230±112 | 1096±24 | | $k$=0.306 |
| FBS | | | 2375±177 | 818±80 |
| $10^{-4}$ $M$ | | | 6671±230 | 4371±154 |
| $10^{-5}$ $M$ | | | 5883±217 | 3231±91 |
| $10^{-6}$ $M$ | | | 5600±46 | 3043±187 |
| $10^{-7}$ $M$ | | | 4255±132 | 1504±138 |
| DMAB control | 2069±120 | 784±32 | | $k$=0.328 |
| FBS | | | 1307±76 | 519±12 |
| $10^{-4}$ $M$ | | | 1978±77 | 744±21 |
| $10^{-5}$ $M$ | | | 5293±26 | 1976±89 |
| $10^{-6}$ $M$ | | | 4356±96 | 1510±14 |
| $10^{-7}$ $M$ | | | 2167±107 | 782±8 |
| NF control | 2082±33 | 649±15 | | $k$=0.301 |
| FBS | | | 1304±76 | 419±6 |
| $10^{-4}$ $M$ | | | 1934±142 | 581±42 |
| $10^{-5}$ $M$ | | | 2420±69 | 765±7 |
| $10^{-6}$ $M$ | | | 2403±100 | 710±33 |
| $10^{-7}$ $M$ | | | 2651±136 | 859±2 |
| PB control | 2706±98 | 504±15 | | $k$=0.191 |
| FBS | | | 1736±112 | 321±2 |
| $3\times10^{-3}$ $M$ | | | 2929±35 | 612±8 |
| $10^{-3}$ $M$ | | | 3695±162 | 741±13 |
| $10^{-4}$ $M$ | | | 3033±135 | 607±48 |
| $10^{-5}$ $M$ | | | 3291±42 | 609±49 |

sensitive to various types of carcinogens; 2) able to detect genotoxicity and mitogenicity of chemicals at different doses; 3) simple with a minimum of radioactive discharge; and 4) able to provide general information on DNA synthesis (general indices for harmonization of genotoxicity, mitogenicity and initiating activities of chemicals). The CDR described here is one approach to fulfilling these requirements.

Previously, Hsia *et al.*[42] reported a method using polyvinyl chloride filters for DNA retention. The CDR utilization of filters facilitates cell culture, nucleation and collection of DNA, providing: 1) a rapid and simple method for measurement of DNA synthesis based on complete nucleation of hepatocytes; 2) simultaneous estimation of UDS and RDS based on subgroups with or without GA and with or without HU; and 3) establishment of general indices of genotoxicity and mitogenicity of chemicals.

**A rapid and simple method for measurement of DNA synthesis based on complete nucleation of rat hepatocytes**

Complete removal of extra-DNA radioactivity with maintenance of the integrity of nuclei is a key process. In CDR, pretreatment with a hypotonic solution brought about complete removal of cytoplasmic structures by

Table II. DNA Synthesis (cpm)

| Samples | ΔRDS | ΔUDS | Total RDS | Total UDS | Total DNA synthesis |
|---|---|---|---|---|---|
| $AFB_1$ | | | | | |
| solvent control | — | — | 2168±135 | −66±79 | 2102±58 |
| $10^{-5}$ *M* | −1218±178 | 3058±108 | 950±189 | 2992±155 | 3942±52 |
| $10^{-6}$ *M* | −1389±139 | 2483±81 | 779±205 | 2367±101 | 3145±112 |
| $10^{-7}$ *M* | −977±227 | 1623±174 | 1192±165 | 1557±129 | 2748±40 |
| $10^{-8}$ *M* | 36±51 | 525±19 | 2204±116 | 459±65 | 2662±51 |
| DMBA | | | | | |
| solvent control | — | — | 5374±169 | 20±48 | 5394±121 |
| $10^{-4}$ *M* | −1152±210 | 1348±123 | 4222±96 | 1369±78 | 5590±74 |
| $10^{-5}$ *M* | −1147±164 | 578±10 | 4227±33 | 598±39 | 4825±46 |
| $10^{-6}$ *M* | −264±115 | 88±34 | 5109±78 | 109±39 | 5218±39 |
| $10^{-7}$ *M* | 397±161 | −47±42 | 5771±111 | −27±69 | 5744±58 |
| DEN | | | | | |
| solvent control | — | — | 2622±100 | 65±8 | 2687±102 |
| $10^{-4}$ *M* | 398±123 | −44±87 | 3020±102 | 21±89 | 3041±148 |
| $10^{-5}$ *M* | 104±16 | 58±22 | 2726±110 | 123±30 | 2849±127 |
| $10^{-6}$ *M* | 57±138 | 234±24 | 2679±234 | 299±21 | 2978±215 |
| $10^{-7}$ *M* | 289±40 | 59±13 | 2911±72 | 123±20 | 3035±91 |
| 2-AAF | | | | | |
| solvent control | — | — | 3063±126 | 167±15 | 3230±112 |
| $10^{-4}$ *M* | 240±410 | 3202±209 | 3302±296 | 3368±194 | 6671±230 |
| $10^{-5}$ *M* | 745±99 | 1908±82 | 3808±225 | 2075±82 | 5883±217 |
| $10^{-6}$ *M* | 609±307 | 1762±262 | 3671±208 | 1928±250 | 5600±46 |
| $10^{-7}$ *M* | 888±346 | 138±228 | 3950±321 | 305±226 | 4255±132 |
| DMAB | | | | | |
| solvent control | — | — | 1971±150 | 98±42 | 2069±120 |
| $10^{-4}$ *M* | −78±91 | −13±43 | 1892±86 | 86±10 | 1978±77 |
| $10^{-5}$ *M* | 3117±235 | 107±141 | 5088±99 | 205±123 | 5293±26 |
| $10^{-6}$ *M* | 2395±25 | −108±21 | 4366±128 | 9±33 | 4356±96 |
| $10^{-7}$ *M* | 154±35 | −56±37 | 2125±153 | 42±46 | 2167±107 |
| NF | | | | | |
| solvent control | — | — | 2050±53 | 32±26 | 2088±33 |
| $10^{-4}$ *M* | −115±195 | −33±32 | 1935±145 | −1±6 | 1934±142 |
| $10^{-5}$ *M* | 316±160 | 22±62 | 2366±107 | 54±39 | 2420±69 |
| $10^{-6}$ *M* | 372±47 | −51±35 | 2422±98 | −19±11 | 2403±100 |
| $10^{-7}$ *M* | 511±181 | 57±41 | 2562±195 | 89±60 | 2651±136 |
| PB | | | | | |
| solvent control | — | — | 2722±119 | −16±27 | 2706±98 |
| $3\times10^{-3}$ *M* | 143±84 | 80±18 | 2865±38 | 65±9 | 2929±35 |
| $10^{-3}$ *M* | 929±82 | 59±30 | 3651±199 | 43±40 | 3695±162 |
| $10^{-4}$ *M* | 277±52 | 49±57 | 3000±159 | 33±58 | 3033±135 |
| $10^{-5}$ *M* | 593±167 | −8±76 | 3315±64 | −24±58 | 3291±42 |

pepsin. Most non-specific radioactivity is contained in mitochondria and microsomes. For CDR, 3 $\mu$m pores are used for separation of nuclei from these organelles, since more than 99% of nuclei of rat heapatocytes are larger than 5 $\mu$m while mitochondria are smaller than 2 $\mu$m.[43] Only 10 out of $5\times10^5$ cells (less than 0.0002%) were lost. This method allowed non-specific radioactivity to be markedly reduced, enabling more than 100 specimens to be examined within 2 days.

**Simultaneous estimation of UDS and RDS based on 4 subgroups with or without carcinogen and with or without HU** Simultaneous estimation of UDS and RDS was earlier attempted by Furihata[44] and Ohsawa *et al.*,[45] by applying HU to an *in vivo* pyloric mucosa short-term assay system with routine alkaline elution procedures, but proved unsuccessful with hepatocytes because of difficulties in inhibiting RDS.[44,45] TDS during incubation with HU has been regarded as UDS.[42] In this study, HU suppressed RDS to 35% and all chemicals tested affected RDS. $AFB_1$ and DMBA reduced cell proliferation, while 2-AAF, DMAB and DEN induced an increase, indicating that more attention should be paid to this source of

Table III. Indices (%)

| Samples | Proliferation index | Repair index | Total synthesis | Induction specificity | ΔRDS effect | Total efficiency |
|---|---|---|---|---|---|---|
| $AFB_1$ | | | | | | |
| solvent control | 100±6 | −3±3 | 103±4 | — | — | −3±3 |
| $10^{-5}$ *M* | 44±8 | 139±14 | 188±7 | −0.40±0.05 | −80±15 | 60±10 |
| $10^{-6}$ *M* | 36±8 | 110±10 | 150±3 | −0.57±0.04 | −73±12 | 38±5 |
| $10^{-7}$ *M* | 55±8 | 72±7 | 131±3 | −0.59±0.07 | −34±9 | 39±5 |
| $10^{-8}$ *M* | 102±3 | 21±4 | 127±2 | 0.07±0.10 | 0±1 | 22±5 |
| DMBA | | | | | | |
| solvent control | 100±3 | 0±1 | 100±1 | — | — | 0±1 |
| $10^{-4}$ *M* | 79±3 | 25±1 | 104±2 | −0.85±0.08 | −5±1 | 20±0 |
| $10^{-5}$ *M* | 79±2 | 11±1 | 90±3 | −1.98±0.25 | −2±0 | 9±1 |
| $10^{-6}$ *M* | 95±2 | 2±1 | 97±2 | −2.98±0.60 | 0±0 | 2±1 |
| $10^{-7}$ *M* | 107±3 | 0±1 | 107±3 | −79.8±105 | 0±0 | 0±1 |
| DEN | | | | | | |
| solvent control | 100±4 | 2±0 | 98±0 | — | — | 2±0 |
| $10^{-4}$ *M* | 115±5 | 1±3 | 113±3 | −2.08±4.04 | 0±0 | 1±4 |
| $10^{-5}$ *M* | 104±1 | 5±1 | 106±1 | 1.99±0.53 | 0±0 | 5±1 |
| $10^{-6}$ *M* | 102±5 | 11±1 | 111±4 | 0.30±0.61 | 0±0 | 12±1 |
| $10^{-7}$ *M* | 111±2 | 5±1 | 113±2 | 5.30±1.66 | 0±0 | 5±1 |
| 2-AAF | | | | | | |
| solvent control | 100±4 | 5±1 | 95±1 | — | — | 5±1 |
| $10^{-4}$ *M* | 108±14 | 110±3 | 207±12 | 0.08±0.13 | 9±14 | 119±15 |
| $10^{-5}$ *M* | 124±2 | 68±4 | 182±3 | 0.39±0.05 | 15±1 | 84±5 |
| $10^{-6}$ *M* | 120±10 | 63±7 | 174±6 | 0.38±0.23 | 11±5 | 75±6 |
| $10^{-7}$ *M* | 129±12 | 10±7 | 132±4 | −0.95±4.93 | 0±2 | 12±9 |
| DMAB | | | | | | |
| solvent control | 100±8 | 5±2 | 95±2 | — | — | 5±2 |
| $10^{-4}$ *M* | 96±4 | 4±1 | 96±2 | −5.24±3.39 | 0±0 | 4±1 |
| $10^{-5}$ *M* | 260±23 | 10±6 | 257±13 | −9.50±62.9 | 7±9 | 25±13 |
| $10^{-6}$ *M* | 222±10 | 0±2 | 211±7 | −23.1±4.72 | −7±2 | −1±3 |
| $10^{-7}$ *M* | 108±2 | 2±2 | 105±1 | −9.70±10.7 | 0±0 | 3±3 |
| NF | | | | | | |
| solvent control | 100±3 | 2±1 | 98±1 | — | — | 2±1 |
| $10^{-4}$ *M* | 95±10 | 0±0 | 93±8 | −5.71±14.1 | 0±0 | 0±0 |
| $10^{-5}$ *M* | 116±8 | 3±2 | 116±5 | −0.67±5.36 | 0±1 | 3±2 |
| $10^{-6}$ *M* | 118±2 | −1±1 | 115±3 | −33.0±39.1 | 0±0 | −1±1 |
| $10^{-7}$ *M* | 125±9 | 4±3 | 127±7 | 69.1±90.4 | 1±0 | 5±3 |
| PB | | | | | | |
| solvent control | 100±4 | −1±1 | 101±1 | — | — | −1±1 |
| $3\times10^{-3}$ *M* | 105±3 | 2±0 | 108±3 | 2.09±1.43 | 0±0 | 3±0 |
| $10^{-3}$ *M* | 134±2 | 2±1 | 136±2 | 19.6±8.31 | 1±0 | 2±2 |
| $10^{-4}$ *M* | 110±2 | 1±2 | 112±4 | −41.5±71.5 | 0±0 | 1±2 |
| $10^{-5}$ *M* | 122±7 | −1±2 | 122±6 | 6.20±9.39 | 0±1 | −1±3 |

error in detection of UDS using liquid scintillation, even in the early phase of initiation. With the CDR approach $UDS_1$ (base line UDS) is never assumed to be zero, and coefficients *k*, *l* (inhibiting ratio of RDS and UDS by HU) are precisely estimated.

**Establishment of general indices of genotoxicity and mitogenicity of chemicals** Estimation of DNA synthesis may also be influenced by the applied amount of DNA and the sensitivity of the scintillation counter used. In this study, the range of $RDS_1$ (base line RDS) was between 1900 and 5400 cpm. The amount of DNA synthesis is therefore better expressed as a percentage of $RDS_1$ ($\%RDS_1$), and the indices generated with the present approach allow more reliable identification of carcinogens and classification into genotoxic or non-genotoxic categories.

Hepatocytes exposed to carcinogens are known to repair damaged DNA during extended $G_1$. Total RDS in the $10^{-5}$ *M* $AFB_1$ case was larger than that with $10^{-6}$ *M* $AFB_1$, suggesting that the reduction of RDS by exposure to $10^{-6}$ *M* $AFB_1$ may not directly depend on cytotoxicity or cell death, but rather on extension of the $G_1$ phase. In

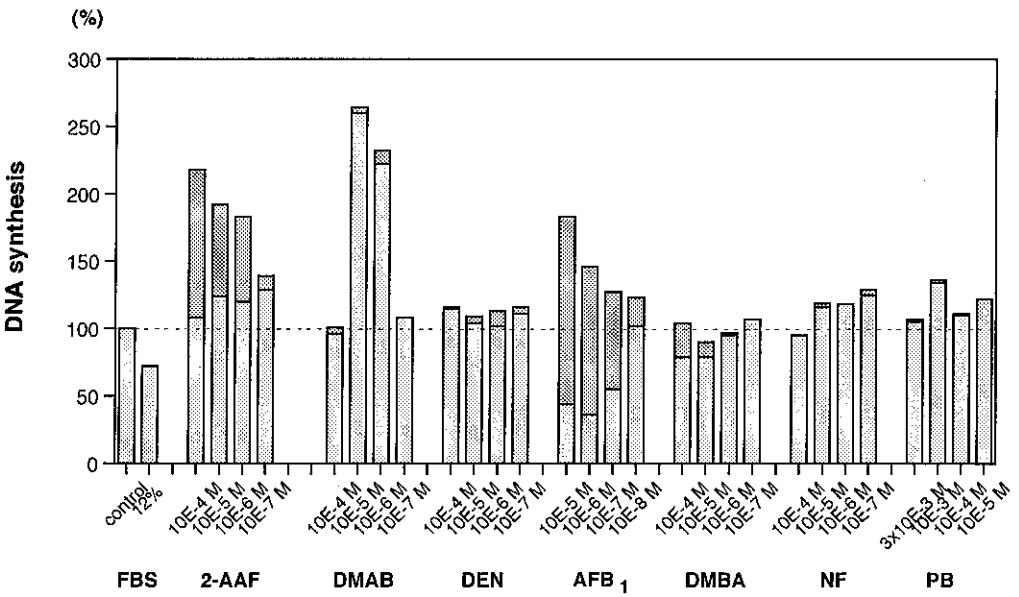


Fig. 4. Induction of UDS and RDS by genotoxic or non-genotoxic agents. A variety of induction patterns was observed. Note the induction or reduction of cell proliferation. □ proliferation index; ▨ repair index.

the present study, $AFB_1$ and DMBA reduced cell proliferation.

Of interest is the fact that DMAB, 2-AAF and DEN induced cell proliferation within the 4 h of the present experiment. It is well known that partially hepatectomized rat liver and primary-cultured rat hepatocytes take 12 h to progress into S phase, and no growth factors induce proliferation of hepatocytes within 12 h of isolation.[46–48] 2-AAF, DMAB and DEN induce cell proliferation in non-proliferating rat hepatocytes prior to the genetically scheduled period, indicating that mitogenic carcinogens, such as these compounds, may affect the regulation of the cell-cycle and allow the hepatocytes to enter S phase.

The role and significance of cell proliferation in carcinogenesis has been well analyzed.[33–38, 48–51] Kaufmann *et al.* characterized hepatocellular proliferation and DNA damage during the initiation phase of rat hepatocarcinogenesis and reported that cell-cycle-dependent variation in sensitivity to initiation of hepatocarcinogenesis is partly related to efficient removal of potentially carcinogenic lesions from DNA during extended $G_1$.[48] A similar role of DNA damage, DNA repair and cell proliferation in other carcinogenesis models was also postulated.[49] From these reports it can be concluded that S phase cells are most sensitive to initiation; they may not complete DNA repair, so that damage remains after replication.[48]

We hypothesize that total RDS is related to the number of target cells of DNA damage, and total UDS corresponds to the extent of DNA damage, in line with Kaufmann's concept[48] since proliferation reduces the possibility of DNA repair. Induction of cell proliferation by carcinogens may play an important role in initiation as well as in the promotion stage of hepatocarcinogenesis. The total extent of unexcised DNA damage remaining after replication in one cell culture insert could be estimated as a synergism between the extent of DNA damage and the number of target cells,

$$\begin{aligned}&\text{Total extent of unexcised DNA damage}\\&=r\times(\text{extent of DNA damage})\times(\text{number of target cells})\\&=r\times(p\times(\text{total UDS}))\times(q\times(\text{total RDS}))\\&=p\times q\times r\times(\text{total UDS})\times(\text{total RDS})\ (\text{cpm}^2)\end{aligned}\qquad[16]$$

($p$, $q$, $r$: coefficients)

Thus, an index for unexcised DNA damage may also be expressed as follows,

$$\text{Index for unexcised DNA damage}=i\times\frac{\text{total UDS}}{\text{RDS}_1}\times\frac{\text{total RDS}}{\text{RDS}_1}\quad(i\text{: coefficient})\qquad[17]$$

A novel index "total efficiency" is defined as follows,

$$\text{Total efficiency}=\frac{\text{total RDS}}{\text{RDS}_1}\times\frac{\text{total UDS}}{\text{RDS}_1}\times100(\%)\qquad[18]$$

The relation between "Index for unexcised DNA damage" and "total efficiency" is,

$$\text{Index for unexcised DNA damage}=\frac{i}{100}\times\text{total efficiency}$$

($i$: coefficient) [19]

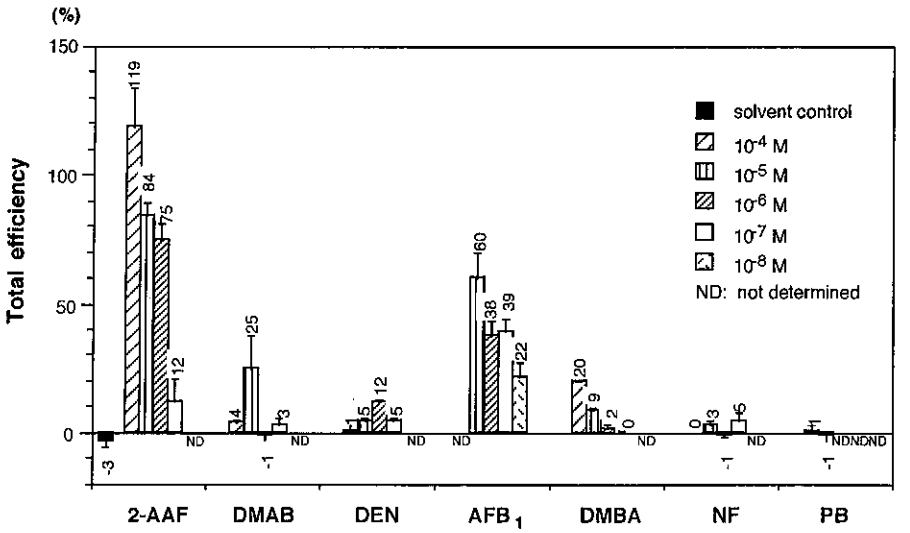


Fig. 5. Total efficiency reflects the extent of effective DNA damage induced by each chemical.

The last expression indicates that "total efficiency" represents the initiating activity of chemicals. The most effective concentration of 2-AAF leaving DNA damage may be $10^{-4}$ *M* (Fig. 5). Although $AFB_1$ was more genotoxic than 2-AAF at any concentration, the mitoinhibitory effect of $AFB_1$ may generate damage less effectively than 2-AAF. Care should be taken in the interpretation of expression [18], however, because many factors including intake, absorption, metabolism, intraplasmic density of chemicals and heterogeneity of DNA repair[52] will affect fixation of DNA damage *in vivo*. For example, non-random distribution of $O^6$-methylguanine has been referred to by Mironov *et al.*[53] Nevertheless, such an index of unexcised DNA damage does suggest a means for the prediction of initiating activity of chemicals. Our findings are basically in accordance with the ideas of Cohen and Ellwein.[33, 34]

Relative sizes of DNA-repair sites (DRS) can also be calculated from the combination of liquid scintillation and autoradiography data. UDS in one cell culture insert consists of the radioactivities of all grains while the radioactivity of one grain corresponds to that of one DNA-repair site. Therefore,

$$\text{DRS} = \text{Mean size of DNA-repair site} = s \times \text{UDS/total number of grains (/well)} = t \times \frac{\text{repair index}}{\text{mean No. of grain (/nucleus)}} \quad (\%/\text{grain}) \quad [20]$$

(*s*, *t*: coefficients)

The expression [20] indicates whether long-patch repair or short-patch repair is performed. Modification of the method should make it possible to express DRS in terms of base pair units.

Evidence that regulation of the cell cycle may underly the intracellular mechanisms of carcinogenesis continues to accumulate. Recently, El-Deiry *et al.*[54] and Xiong *et al.*[55] reported a 21 kilodalton protein that is elicitated by *p53* and blocks cell progression through the cell-cycle. Some direct interaction between carcinogens and cellular proteins may exist, as indicated in the case of 2-AAF, which was shown previously to interact specifically with a target protein, a liver fatty acid binding species, and to modulate the growth of hepatocytes.[56–58]

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