Effects of Variations in Nucleoside Pool Sizes on Comparisons of the Incorporation of $[^3H]$Thymidine into Isolated Rat Liver Cells

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ABSTRACT

Isolated rat liver cells have been used to measure DNA repair synthesis induced by ultraviolet light and aflatoxin. Age, sex, and diet of the rats were found to influence DNA repair in rat liver cells, as measured by $[^3H]$thymidine uptake by the cells. These effects were found to be due to variations in the thymidine pool size and should serve to indicate that high-specific-activity thymidine (i.e., low nucleoside concentrations) is subject to artifacts generated by pool size variations.

INTRODUCTION

Rat liver cells may be isolated by liver perfusion (1), and the use of such cells has been advocated for the study of DNA repair synthesis in response to carcinogens (18). Incorporation of $[^3H]$thymidine into the nucleus as a method of assessing DNA repair synthesis has been suggested as an ideal method for a quick measure of repair after insult by some suspected DNA-damaging agent (15). Irradiation by UV (254 nm) has been shown to initiate such damage and repair (14) and was used as a convenient carcinogenic agent. During this work, it has been found that variables such as sex, age, and diet of the animals from which cells are derived have an influence on the unscheduled incorporation of $[^3H]$thymidine by the cell DNA after carcinogen attack. These effects are probably explained by variation in the intracellular thymidine pool.

Similar observations have been repeated by Hell et al. (8), who discovered artifacts in high-specific-activity $[^3H]$thymidine uptake by Ehrlich ascites cells after exposure to X-rays. The irradiation apparently caused dilution of the thymidine pool by release of DNA precursors. Cleaver and Holford (4) showed, in addition, that pool size is proportional to the square root of the thymidine concentration in the medium at less than saturating concentrations, so that incorporation of $[^3H]$thymidine into DNA is directly proportional to pool size. Consequently, variations in very low extracellular concentrations of thymidine in the animal prior to cell preparation may cause variations in the pool that are great enough to affect $[^3H]$thymidine incorporation into DNA.

Lambert et al. (10) have recently published their finding that, with human peripheral leukocytes, there is a decrease with age in the capacity for UV-induced DNA repair synthesis. These workers found that the decrease in uptake of $[^3H]$thymidine with age of the human cell donors was not due to an increase in dTTP pool size. The results presented here using rat liver cells suggest that variations in uptake of $[^3H]$thymidine with age and sex are due to variation in thymidine pool size.

MATERIALS AND METHODS

Rats. Male and female hooded Wistar rats were killed at 10 to 12 weeks of age, except in the experiment where the effect of age was studied. Where required, castration was carried out 2 weeks prior to the preparation of cells.

Diets. Pelleted diets were purchased from W. Charlick Ltd., Adelaide, South Australia. Diet 1 consisted of a standard laboratory pellet of the following composition: protein, 17.5%; fat, 3.5%; fiber, 5.5%; NaCl, 0.5%; carbohydrate, 72%; vitamin A, 9900 IU/kg; vitamin D3, 1300 IU/kg; vitamin B, 4 mg/kg; vitamin E, 24 IU/kg; vitamin K, 2 mg/kg; vitamin B12, 11 μg/kg; pantothenic acid, 11 mg/kg; nicotinic acid, 44 mg/kg; and chlorotetracycline, 12 ppm. Diet 2 consisted of a standard laboratory pellet of the following composition: protein, 21%; digestible energy, 13 MJ/kg; and vitamins and minerals as recommended by the American Institute of Nutrition (9). Diet 3 consisted of a laboratory-prepared diet (30% protein) (11). Diet 4 consisted of a laboratory-prepared diet (no protein) (11).

Isolation and Incubation of Liver Cells. Rats were anesthetized with sodium pentobarbitral at the same time each morning, and the cells were prepared by liver perfusion with 0.05% collagenase (Worthington Biochemical Corp., Freehold, N. J.) in Hank's balanced salt solution, without added calcium, by the method of Berry and Friend (1). Directly after perfusion, the isolated cells were washed and suspended in Hank's balanced salt solution at a density of 4 to 6 million cells/ml. Glass Petri dishes, 4 cm in diameter, were placed in an atmosphere of carbogen (95% CO2:5% O2), and a cell suspension (2.5 ml) was added to each dish. Individual dishes were exposed to UV (254 nm) at 0.9 J/sq m/sec. As an alternative carcinogen, aflatoxin B1 (Calbiochem-Behring Corp., La Jolla, Calif.), was incorporated in the incubation medium in experiments where noted. In all experiments, control incubations were carried out under the same conditions but without treatment with the DNA-damaging agent to determine the background incorporation of $[^3H]$thymidine. Dose response uptakes of $[^3H]$thymidine were recorded as the difference in cpm between treatments and controls.

Triplicate samples (0.5 ml) of cell suspensions were added to incubation vessels, gassed with carbogen, sealed with rubber stoppers, and incubated at 37°C for 1.5 hr in a shaking water bath at 90 oscillations/min. The glass incubation vessels (2.5 × 5.0 cm) contained in addition, Eagle’s minimal essential medium for suspension (1 ml containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), 1 μCi $[^3H]$-thymidine (47 Ci/mmol; The Radiochemical Centre, A nerzhou, United Kingdom), and 5 mm hydroxyurea (6)). Any possible thymidine content of the serum was removed by passage through chromatography charcoal (BDH Pharmaceuticals Ltd., Poole, England).

Incubations were stopped by cooling in ice and adding 1 ml...
ice-cold magnesium- and calcium-free phosphate-buffered saline (0.2 g KCl; 0.2 g KH₂PO₄; 8 g NaCl; 1.15 g Na₂HPO₄·2H₂O in 1000 ml H₂O) containing 0.5 mg thymidine per ml. After 3 washes in phosphate-buffered saline (5 ml), cells were suspended in 1% sodium citrate (1 ml) and fixed with 2.5 ml ethanol:acetic acid (3:1), following the washing procedure of Stich and San (17), who used this method prior to autoradiography to estimate [³H]thymidine incorporation into the nucleus. Previous experience indicated that this method results in lower background counts than washing cells in cold 5% trichloroacetic acid. The fixed cells were washed in ethanol (5 ml) before solution in NCS tissue solubilizer (0.3 ml) and toluene scintillation medium (5 ml) for liquid scintillation counting. Preliminary experiments (not reported here) indicated that scintillation counting gave a more accurate assessment of [³H]thymidine incorporation than that provided by autoradiography. Isolation of DNA from the cells by the technique of Schneider and Greco (16) revealed that, in both the background counts and treated cells, 80% of the counts incorporated into the cells were located in the DNA fraction. We therefore considered that the net cpm recorded in the cells was a true representation of unscheduled DNA synthesis.

RESULTS

Time of Incubation. Incubation of isolated hepatocytes for increasing periods of time after irradiation with UV (72 J/sq m) produces a maximum uptake of [³H]thymidine after 1.5 hr. Thereafter, all incubations were carried out for 1.5 hr.

Effect of Sex of Rat. Male and female rats and castrates of both sexes were maintained on ad libitum Diet 1. Liver cells prepared from these animals were exposed to UV and the dose response is shown in Chart 1. It can be seen that the cells from castrated male rats had a greater [³H]thymidine uptake than those of males, and cells from castrated female rats had a lower uptake of [³H]thymidine than those of females. Exposure of cells from male and female rats to increasing doses of aflatoxin B₁, as an alternative chemical carcinogen also caused greater [³H]thymidine uptake by cells from females than those from males. The increasing concentration of aflatoxin B₁ has a toxic effect on the cells, which is shown by the dose-response curve (Chart 2).

Effect of Diet of Rat. During the course of these experiments over an extended period of time, it was found that variations in [³H]thymidine uptake after UV treatment of the cells were coincident with changing batches of rat feed from the same manufacturer. Dietary protein levels are known to influence levels of some enzymes in the liver (11), although it was considered unlikely that manufacturing variations would be sufficient to cause this effect. Incorporation of [³H]thymidine by the liver cells could be affected either by variations in DNA synthesizing enzymes or by the intracellular thymidine pool size. Examination of the pool size may be carried out by dilution of [³H]thymidine with increasing molarity of thymidine in the medium, provided that the concentrations are not high enough to cause inhibition of DNA synthesis (2, 5, 7, 12, 13).

To test the proposition that extreme variations in dietary protein (which is not suggested to have occurred in the commercial feed) may alter levels of unscheduled DNA synthesis, 2 groups of 4 rats (6 weeks old) were fed Diets 3 and 4 for 9 days prior to liver perfusion. Liver cells exposed to a single dose of UV (72 J/sq m) and then incubated with increasing levels of thymidine in the medium produced the [³H]thymidine uptake shown in Chart 3. It can be seen that the cells from rats fed the no-protein diet maintained a higher uptake of [³H]thymidine, even when thymidine pools are diluted. The results shown in Table 1 indicate that this is true for both normal DNA synthesis in the controls and the unscheduled DNA synthesis after UV irradiation.
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whether this effect was due to variation of thymidine pool size with age, groups of 5 animals at 4 weeks and 12 weeks of age were used to prepare cells for thymidine pool dilution incubations. The results (Chart 5) show that the [3H]thymidine uptake is higher in younger animals when the cells are incubated in the presence of [3H]thymidine of highest specific activity but that, when the radiolabeled compound is diluted to 0.1 µM concentration in the medium, the uptake of cells of both young and old rats is the same.

DISCUSSION

Lambert et al. (10), using peripheral human leukocytes, found a decrease with age in the capacity for UV-induced DNA repair synthesis. Measurement of dTTP pools in the isolated peripheral lymphocyte fraction showed that the effect was not

The observed differences in [3H]thymidine uptake of liver cells from male and female rats may be due to a thymidine pool size effect. Cells from 2 groups of 4 male and female rats (aged 10 weeks) maintained on the same batch of Diet 1 were exposed to a single dose of UV (72 J/sq m) and [3H]thymidine uptake measured with increasing concentration of substrate thymidine. The difference in uptake observed with the highest-specific-activity [3H]thymidine is not confirmed with isotope dilution (Chart 4).

Effect of Age of Rat. Male rats of various ages were maintained on Diet 2 and used to prepare liver cells for DNA repair response to UV. There is a decline of the [3H]thymidine uptake by liver cells from 4 weeks of age to 12 weeks of age, and no further decline is evident at 20 weeks of age. To determine
due to an increase in pool size with age. Contrary to this, we have found that, with isolated rat liver cells, the decline in UV-induced DNA repair with increasing age is nullified by saturating the thymidine pool. Further, rat liver cells from mature females were found to have a greater DNA repair compared to males, with castrates of both sexes intermediate between the 2 groups, and these results were also nullified by saturating the thymidine pool.

There are variations in $[^3H]$thymidine uptake of rat liver cells due to an unknown variable component of the commercial pelleted diet, and it is suggested that the observed effect on DNA repair of the sex of the rat may be due to the greater food intake of males, resulting in an increase in thymidine pools in the liver cells. The protein content of rat feed does have an effect on the DNA repair capacity of the isolated liver cells, but this variable is not negated by saturation of the thymidine pool.

Change in thymidine pool size affects not only DNA repair synthesis but also normal DNA synthesis, as may be expected. Control of DNA synthesis via control of precursor pools is a well-established finding. This is emphasized by these results, together with those of Lambert et al. (10), and shows that comparative studies of DNA repair between cells derived from individuals are therefore suspect unless substantiated by direct determination of nucleotide pools in the cell or by isotope dilution. Similarly, comparison carried out within a cultured cell line would be valid only if cultural conditions affecting nucleotide pools are identical.

It may be noted that Lambert’s experiments with human peripheral leukocytes (10) were carried out with 2 $\mu$M thymidine in the medium, which is greater than the saturating concentrations used in our liver cell experiments. However, as Cleaver (3) points out, some of the results obtained with peripheral lymphocytes are different from those obtained with other cell types, and Cleaver suggests that this is probably due to differences in pool sizes in various cell types. It is also possible that there are differences in permeability to thymidine in various cell types, so that different saturating concentrations are required. These factors may explain the differences in suppression of normal DNA synthesis by hydroxyurea; i.e., over 90% for human peripheral leukocytes (10) and 66% for isolated rat liver cells, as reported by Williams (19) and confirmed in our experiments.

Measurement of the repair of DNA by incorporation of $[^3H]$thymidine after carcinogen-induced damage has often been used as a measure of the potency of a carcinogen. These results indicate that it is not valid to reach general conclusions from experiments with a single cell type. Diet of the rat is an important factor influencing UV-induced $[^3H]$thymidine uptake by isolated rat liver cells, and all conclusions should be supported by estimation of nucleotide pool sizes.

REFERENCES

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