Bull. Environ. Contam. Toxicol. (1985) 34:340–348

Bulletin of Environmental Contamination and Toxicology

Increased Response of the Rainbow Trout Gonad Cell Unscheduled DNA Repair Assay

Douglas G. Walton, Alfred B. Acton, and Hans F. Stich

Environmental Carcinogenesis Unit, British Columbia Cancer Research Center, 601 West 10th Avenue, Vancouver, B.C. V5Z 1L3, Canada

Epstein (1974) and Heidelberger (1975) suggest that 60-90% of all human cancers involve a chemical carcinogen. Many of these chemicals have been isolated from discharges to the aquatic environment (Dunn and Stich 1976; Kraybill 1977; Payne and Rahimtula 1981), and examination of contaminated areas has shown a relationship between enhanced levels of known carcinogens and neoplastic disease in fish (Brown et al. 1973; Smith et al. 1979; Black et al. 1980, 1982). A chemical agent etiology is further supported by the production of tumors in fish following exposure to carcinogens (Ashley 1970; Sato et al. 1973; Pliss and Khudoley 1975), by the ability of fish to metabolically activate chemical carcinogens (Balk et al. 1982; Egaas and Varanasi 1982; Varanasi et al. 1982), and by the binding of these active metabolites to fish DNA (Ahokas et al. 1979; Varanasi et al. 1981; Bailey et al. 1982).

A number of *in vivo* test systems for studying genotoxic effects of chemicals on aquatic organisms have been proposed (Kligerman et al. 1975; Kligerman 1979; Pesch and Pesch 1980; Dixon and Clarke 1982; Harrison and Jones 1982; Hooftman and de Raat 1982), but there has been comparatively little research on *in vitro* systems (Barker and Rackman 1979; Slooff and van Kreijl 1982; Walton et al. 1983).

In mammalian test systems, the UDS technique has proven to have *in vivo* and *in vitro* applications and responds to many classes of carcinogens (Mitchell et al. 1983). UDS is also of practical significance, since failure to repair damaged DNA is linked to increased tumor incidence (Cleaver and Bootsma 1975). Studies by Ishikawa et al. (1978) *in vivo* and by Woodhead et al. (1980) and Walton et al. (1983) *in vitro* have shown limited UDS in fish cells independent of the cell line's tissue or species of origin following chemical mutagen exposure. In this paper, we present data showing how the *in vitro* UDS assay with rainbow trout gonad (RTG) cells may be modified to enhance grain production, thus increasing the sensitivity of the assay.

MATERIALS AND METHODS

RTG-2 cells (passage 60), obtained from the American Type Culture Collection, Rockville, MD, were maintained in sealed 75 cm^2 plastic culture flasks (Falcon Plastics) at 18°C in a dry incubator. The cells were cultured in Eagle's minimal essential medium (MEM, Grand Island Biological Co.) supplemented with 10% fetal calf serum, antibiotics (streptomycin sulfate, 29.5 µg/ml; penicillin G, 204 µg/ml; kanamycin, 100 µg/ml; fungizone, 2.5 µg/ml) and sodium bicarbonate (5 ml/800 ml medium).

N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 4-nitroquinoline-1-oxide (4NQO) were obtained from Aldrich Chemical Co., Milwaukee, WI. [Methyl-^{3}H]thymidine (^{3}HTdR, sterile aqueous solution, 25 Ci/mmole specific activity) was from the Amersham Corporation, Amersham, U.K.

Approximately 80,000 cells were seeded onto 10 x 35 mm coverslips in stoppered Bellco Leighton tubes. After 2-3 days in growth medium (MEM plus 10% serum), cell proliferation was arrested by placing the cultures into arginine-deficient medium (ADM) with 2.5% serum (2.5% ADM) for 4-5 days.

MNNG dissolved directly into 2.5% ADM, while 4NQO required initial dissolving in dimethylsulfoxide (DMSO) prior to dilution with medium. ^{3}HTdR was diluted with 2.5% ADM to attain a working concentration of 10 µCi/ml. Cells were simultaneously exposed to mutagen and ^{3}HTdR for the treatment period.

Following exposure, the cells were treated with 0.5% sodium citrate for 10 min and then fixed in ethanol/acetic acid (3:1). Air-dried coverslips were mounted on slides with paraffin wax, processed autoradiographically using Kodak Nuclear Track Emulsion (NTB-3, Eastman Kodak, Rochester, NY), and stained in aceto-orcein. The amount of UDS was determined by scoring the number of grains over nuclei of equal size and staining intensity, and subtracting the background grain count from an equivalent area adjacent to the nucleus. Each data point represents the mean number of silver grains for at least 30 nuclei.

RESULTS AND DISCUSSION

Extending the ^{3}HTdR treatment period (Fig. 1) to 6 hr resulted in higher grain counts. Presumably, a longer treatment period permits more DNA to be repaired, hence ^{3}HTdR incorporation. No toxic effects were observed with the longer ^{3}HTdR exposure. Data point comparisons generally indicate that grain count increases are not proportional to increases in ^{3}HTdR exposure time, probably a consequence of the rapidly falling rate of repair following mutagen exposure (Walton et al. 1983). A longer ^{3}HTdR treatment period following mutagen exposure has also been found to increase grain counts in rat primary hepatocytes (Williams and Laspia 1979).

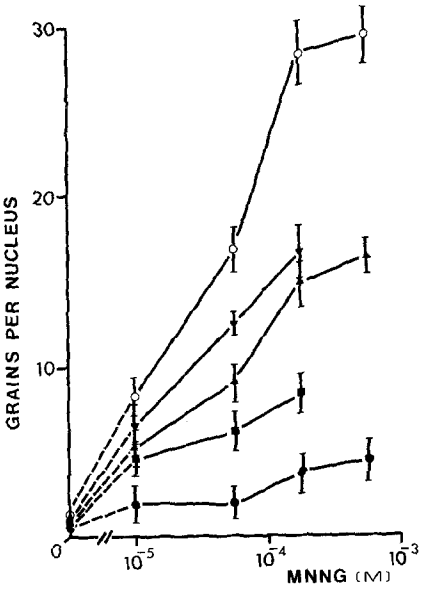

Fig. 1. The effect of increased ^{3}HTdR exposure time (●, 30 min; ■, 60 min; ▲, 90 min; ▼, 180 min; O, 360 min) on UDS in cultured RTG cells following a 30 min MNNG treatment. The assay was conducted at 18°C and emulsion-coated slides kept in light-tight boxes for 18 days prior to development. Mean grain counts are plotted with 99% confidence intervals.

Assay temperature influences grain production (Fig. 2). Conducting the assay over a temperature range of 4 to 25°C indicated maximal UDS at 25°C. Cell killing from the temperature change was not observed during the course of the experiment. Enhanced grain production by elevating the assay temperature to 25°C may result from increased activity of the DNA repair enzymes, since immediate changes in poikilothermic cells (Hazel and Prosser 1974) are more likely to be enzyme-related than, for example, membrane composition alterations (de Torrengo and Brenner 1976), which could affect chemical mutagen or ^{3}HTdR uptake. Observation of RTG cell cultures maintained for several passages at 25°C showed an increase in the number of karyorrhexic nuclei, indicating that the nature of the DNA may be influenced by temperature. Plumb and Wolf (1971) found the optimal temperature for RTG cell growth to be approximately 20°C, with only slightly less growth at 25°C.

Increasing the duration of exposure to the emulsion (Fig. 3) also yielded an increase in nuclear grain count. A longer emulsion exposure period permits more ^{3}HTdR to decay and react with the emulsion to produce more grains over the nucleus. As the half-life of tritium is 12.3 years (Rogers 1979), the rate of decay should be constant over the 30-day exposure period used here. As expected, the results (Fig. 3) show a proportional grain count increase with increase in emulsion exposure time.

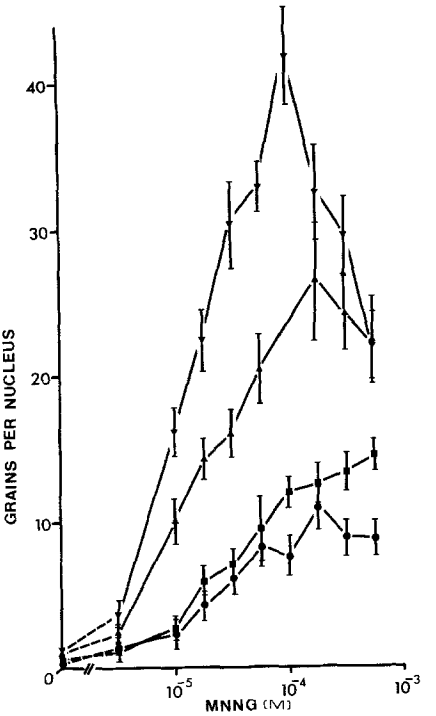

Fig. 2. The UDS increase with assay temperature (●, 4°C; ■, 11°C; ▲, 18°C; ▼, 25°C) in cultured RTG cells exposed simultaneously to ^{3}HTdR and MNNG for 6 hr was examined. The cells were seeded at 18°C and given 48 hr to acclimatize to the new temperature prior to the experiment. The cells were exposed to emulsion for 18 days prior to development. Mean grain counts are plotted with 99% confidence intervals.

Increased grain production was found by lengthening the duration of carcinogen exposure to approximately 30 min (Fig. 4). Between 30 and 60 min there is only a slight increase in grain count, and the slight decrease in counts beyond 60 min may indicate a toxic effect from the mutagen. An increase in rat primary hepatocyte grain counts has also been found with longer mutagen exposure (Williams 1977).

Other factors investigated, including varying the cell division arrest time in 2.5% ADM prior to mutagen exposure, increasing the culture media's fetal calf serum concentration, varying the ^{3}HTdR concentration, and using RTG cells differing by an excess of 30 passages, had little effect on the observed level of UDS.

By conducting the assay at 25°C instead of 18°C, increasing the ^{3}HTdR treatment period from 3 to 6 hr, and extending the emulsion exposure period from 18 to 30 days, grain production in RTG cells can be increased two to threefold (Fig. 5).

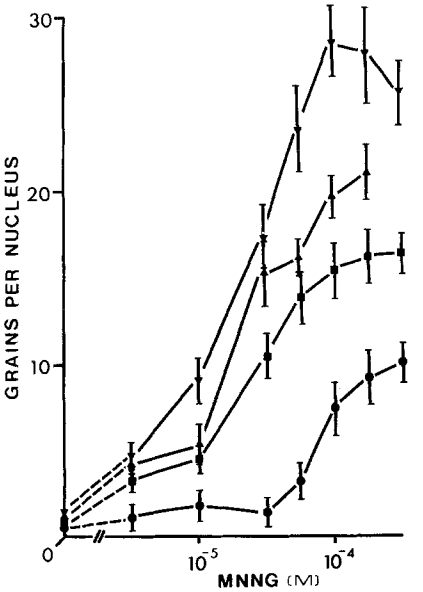

Fig. 3. Following simultaneous exposure to MNNG and ^{3}HTdR at 18°C, coverslips of cultured RTG cells were mounted on slides and exposed to emulsion for varying periods of up to 28 days (●, 7 days; ■, 14 days; ▲ , 21 days; ▼ , 28 days) prior to development. Mean grain counts are plotted with 99% confidence intervals.

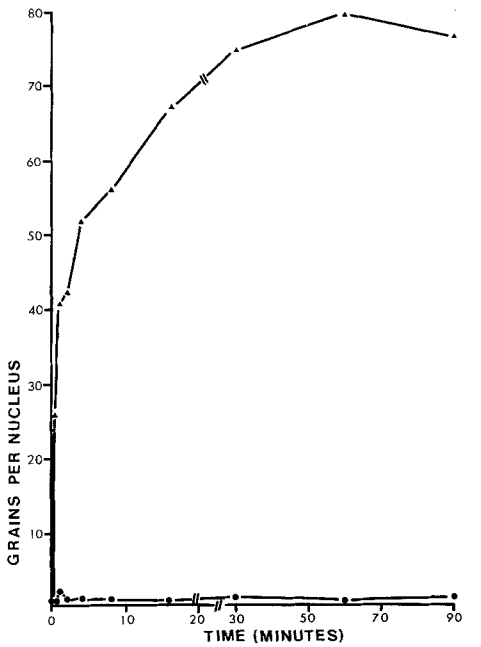

Fig. 4. The UDS increase in cultured RTG cells at 25°C with increased exposure time to $5x10^{-4}$ M 4NQO (▲, experimental; ● , control) is shown. The 4NQO exposure was followed by a 6-hr ^{3}HTdR treatment and a 30-day emulsion exposure.

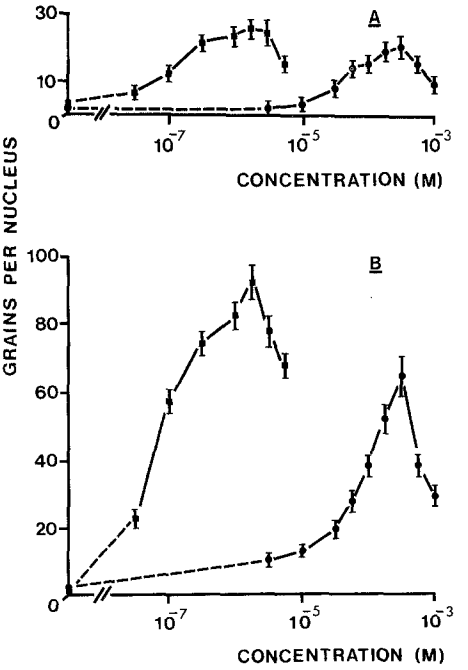

Fig. 5. A comparison of UDS in cultured RTG cells exposed to MNNG (●) or 4NQO (■) utilizing (A) the original experimental conditions (assay at 18°C using a simultaneous 3-hr ^{3}HTdR treatment and 18-day exposure period to emulsion), and (B) the conditions found to enhance grain production (assay at 25°C using a simultaneous 6-hr ^{3}HTdR treatment and 30-day exposure period to emulsion) is shown. Mean grain counts are plotted with 99% confidence intervals.

Background grain counts, observed to increase slightly, were minimized by using a low cell density on each coverslip and by thorough rinsing of the coverslip during the preservation procedure to remove non-incorporated ^{3}HTdR.

Further research will determine whether this enhanced response is sufficient for the RTG DNA repair assay to be a viable genotoxicity testing procedure.

Acknowledgments

These studies were supported by the National Cancer Institute of Canada and by the B.C. Foundation for Non-Animal Research. Dr H.F. Stich is a Research Associate of the National Cancer Institute of Canada. The authors wish to thank Mrs G. Wood for tissue culture assistance, Dr R. San for his comments and encouragement, and Miss R. Johnson for typing the manuscript.

REFERENCES

Ahokas JT, Saarni H, Nebert DW, Pelkonen O (1979) The in vitro metabolism and covalent binding of benzo(a)pyrene to DNA catalysed by trout liver microsomes. Chem-Biol Interact 25: 103-111

Ashley LM (1970) Pathology of fish fed aflatoxins and other anti-metabolites. In: Snieszko SF (ed) A symposium on diseases of fishes and shellfishes. American Fisheries Society, Washington DC, pp 366-379

Bailey GS, Taylor MJ, Selivonchick DP (1982) Aflatoxin B_1 metabolism and DNA binding in isolated hepatocytes from rainbow trout (S. gairdneri). Carcinogenesis 3:511-518

Balk L, DePierre JW, Sundvall A, Rannug U (1982) Formation of mutagenic metabolites from benzo(a)pyrene and 2-aminoanthracene by the S-9 fraction from the liver of the northern pike (Esox lucius): inducibility with 3-methylcholanthrene and correlation with benzo(a)pyrene monooxygenase activity. Chem-Biol Interact 41:1-13

Barker CJ, Rackman BD (1979) The induction of sister-chromatid exchanges in cultured fish cells (Ameca splendens) by carcinogenic mutagens. Mutation Res 68:381-387

Black JJ, Holmes M, Paigen B, Dymerski PP, Zapisek WF (1980) Fish tumor pathology and aromatic hydrocarbon pollution in a Great Lakes estuary. In: Afghan BK, Mackay D (eds) Hydrocarbons and halogenated hydrocarbons in the aquatic environment. Plenum, New York, pp 559-565

Black JJ, Evans ED, Harshbarger JC, Zeigel RF (1982) Epizootic neoplasms in fishes from a lake polluted by copper mining wastes. J Natl Cancer Inst 69:915-926

Brown ER, Hazdra JJ, Keith L, Greenspan I, Kwapinski JBG, Beamer P (1973) Frequency of fish tumors found in a polluted watershed as compared to non-polluted Canadian waters. Cancer Res 33:189-198

Cleaver JE, Bootsma D (1975) Xeroderma pigmentosum: biochemical and genetic characteristics. Ann Rev Genet 9:19-38

Dixon DR, Clarke KR (1982) Sister chromatid exchanges: a sensitive method for detecting damage caused by exposure to environmental mutagens in the chromosomes of adult Mytilus edulis. Mar Biol Lett 3:163-172

Dunn BP, Stich HF (1976) Monitoring procedures for chemical carcinogens in coastal waters. J Fish Res Board Can 33:2040-2046

Egaas E, Varanasi U (1982) Effects of polychlorinated biphenyls and environmental temperature on in vitro formation of benzo(a)-pyrene metabolites by liver of trout (Salmo gairdneri). Biochem Pharmacol 31:561-566

Epstein SS (1974) Environmental determinants of human cancer. Cancer Res 34:2425-2435

Harrison FL, Jones IM (1982) An in vivo sister-chromatid exchange assay in the larvae of the mussel Mytilus edulis: response to 3 mutagens. Mutation Res 105:235-242

Hazel JR, Prosser CL (1974) Molecular mechanisms of temperature compensation in poikilotherms. Physiol Rev 54:620-677

Heidelberger C (1975) Chemical carcinogenesis. Ann Rev Biochem 44:79-121
Hooftman RN, de Raat WK (1982) Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mudminnow Umbra pygmaea by ethyl methanesulphonate. Mutation Res 104:147-152
Ishikawa T, Takayama S, Kitagawa T (1978) Autoradiographic demonstration of DNA repair synthesis in ganglion cells of aquarium fish at various ages in vivo. Virchows Arch B Cell Pathol 28:235-242
Kligerman AD (1979) Induction of sister chromatid exchanges in the central mudminnow following in vivo exposure to mutagenic agents. Mutation Res 64:205-217
Kligerman AD, Bloom SE, Howell WM (1975) Umbra limi: a model for the study of chromosome aberrations in fish. Mutation Res 31: 225-233
Kraybill HF (1977) Global distribution of carcinogenic pollutants in water. Ann NY Acad Sci 298:80-89
Mitchell A, Casciano D, Martin M, Robinson D, San R, Williams G, von Halle E (1983) Unscheduled DNA synthesis tests. Mutation Res 123:363-410
Payne JF, Rahimtula A (1981) Water chlorination as a source of aquatic environmental mutagens. In: Khan MAQ, Stanton RH (eds) Toxicology of halogenated hydrocarbons: health and ecological effects. Pergamon, New York, pp 209-221
Pesch GG, Pesch CE (1980) Neanthes arenaciodentata (Polychaeta: Annelida), a proposed cytogenetic model for marine genetic toxicology. Can J Fish Aquat Sci 37:1225-1228
Pliss GB, Khudoley VV (1975) Tumor induction by carcinogenic agents in aquarium fish. J Natl Cancer Inst 55:129-136
Plumb JA, Wolf K (1971) Fish cell growth rates - quantitative comparison of RTG-2 cell growth at 5-25°C. In Vitro 7:42-45
Rogers AW (1979) Techniques of autoradiography. 3rd ed. Elsevier, Amsterdam
Sato S, Matsushima T, Tanaka N, Sugimura T, Takashima F (1973) Hepatic tumors in the guppy (Lebistes reticulatus) induced by aflatoxin B1, dimethylnitrosamine and 2-acetylaminofluorene. J Natl Cancer Inst 50:767-778
Slooff W, van Kreijl CF (1982) Monitoring the rivers Rhine and Meuse in the Netherlands for mutagenic activity using the Ames test in combination with rat or fish liver homogenates. Aquat Toxicol 2:89-98
Smith CE, Peck TH, Klauda RJ, McLaren JB (1979) Hepatomas in Atlantic tomcod Microgadus tomcod (Walbaum) collected in the Hudson River estuary in New York. J Fish Dis 2:313-319
de Torrengo MP, Brenner RR (1976) Influence of temperature on the fatty acid desaturation and elongation activity of fish (Pimelodus maculatus) liver microsomes. Biochim Biophys Acta 424:36-44
Varanasi U, Stein JE, Hom T (1981) Covalent binding of benzo(a)-pyrene to DNA in fish liver. Biochem Biophys Res Commun 103: 780-787

Varanasi U, Nishimoto M, Reichert WL, Stein JE (1982) Metabolism and subsequent covalent binding of benzo(a)pyrene to macromolecules in gonads and liver of ripe English sole (Parophrys vetulus). Xenobiotica 12:417-425
Walton DG, Acton AB, Stich HF (1983) DNA repair synthesis in cultured mammalian and fish cells following exposure to chemical mutagens. Mutation Res 124:153-161
Williams GM (1977) Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res 37:1845-1851
Williams GM, Laspia MF (1979) The detection of various nitrosamines in the hepatocyte primary culture/DNA repair test. Cancer Lett 6:199-206
Woodhead AD, Setlow RB, Grist E (1980) DNA repair and longevity in three species of cold-blooded vertebrates. Exp Gerontol 15: 301-304

Received May 10, 1984; accepted May 25, 1984