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SHORT-TERM GENOTOXICITY SCREENING OF WATER SAMPLES WITH PRIMARY CULTURES OF RAT HEPATOCYTES – PRELIMINARY RESULTS

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Abstract

Primary cultures of adult rat hepatocytes were tested for their suitability as a short-term genotoxicity screening system for surface water samples. Preliminary results obtained with Salzach river water samples indicate a high sensitivity of this system since even at concentrations of 0,1% significantly elevated levels of SCEs and micronuclei are found. Like for studies with known mutagens/carcinogens SCE-induction proved to be the most sensitive cytogenetic marker.

The water samples tested also revealed that a large part of the mutagenicity is lost during storage although there is residual mutagenicity which appears to remain constant. Studies on the genotoxicity of water samples should therefore be performed immediately after sampling to obtain proper values.

Introduction

Primary cultures of adult rat hepatocytes have been shown to be very sensitive indicators for the clastogenic effects of mutagens/carcinogens, i. e. treatment with aflatoxin B_1 led to significantly elevated levels of sister chromatid exchanges (SCE) already at a concentration of 10^{-12} M (1). Compared to other genotoxicity assay systems which utilize the metabolizing activity of liver microsomes (2, 3, 4), this means that the sensitivity is at least 1–2 orders of magnitude higher, and is due to the fact that procarcinogens are metabolically activated by the liver microsome mixed function oxidases and the active metabolite can interact with the hepatocyte genom, whereas in other assay systems the active metabolite has to be delivered to an indicator cell line, a process which results in a dilution of the metabolite concentration.

In view of the high sensitivity of this genotoxicity assay system it was thought of using this system for the detection of the low levels of mutagenic/carcinogenic compounds found in the environment either directly – by treating primary cultures of hepatocytes with samples of suspect water or air – or indirectly – by pla-

cing hepatocytes of animals living in suspect areas in primary culture. Since the genotoxic damage in hepatocytes appears to persistent over longer periods of time (5, 6), continuous exposure should result in an accumulation of damage. The animals thus could function as biological indicators of carcinogen exposure.

Preliminary results for water samples are reported here.

Materials and Methods

Materials

Female Fischer 344 rats were obtained from Charles River Wiga GmbH, Sulzfeld, West Germany, and were maintained in a temperature- and humidity-controlled room with a 12 h light-dark cycle. MEM with Earle's salts and non-essential amino acids and gentamicin were obtained from Grand Island Biological Company, Heilmittelwerke Wien, Vienna, Austria. EGF, collagenase and other cell culture chemicals were purchased from Sigma Chemical Company, Munich, West Germany, and cellulose nitrate filters were from Sartorius Vertriebsgesellschaft mbH, Vienna, Austria.

Hepatocyte isolation and culture

Hepatocytes were isolated from female Fischer 344 rats by the **in situ** two-step collagenase perfusion technique described by Michalopoulos et al. (7). The isolated hepatocytes were plated at a density of 20.000 viable cells/cm² on collagen-coated 60 mm diameter plastic culture dishes as described (8) using 5 ml serum free MEM supplemented with non-essential amino acids and gentamicin (50 μg/ml) and 0,4 mM Ca^{2+} The plates were then incubated at 37 °C, 5% CO_2 and 95% relative humidity. After an incubation period of 3 hours the medium was exchanged for freshly prepared medium supplemented as described above with the further addition of insulin (10^{-7} M). After the medium had been replaced, the cultures were returned to the incubator.

Preparation of water samples and treatment of hepatocyte cultures

Water samples were taken from the river Salzach near the Überfuhrsteg and right before the river Königseeache joins the Salzach (close to the hydroelectric power plant Urstein). In this area the water is contaminated by the waste-waters of several industrial plants.

The water samples were brought to the laboratory, filtersterilized (0,2 μm poresize cellulose nitrate filters) and medium prepared with the sterile water samples.

Approximately 20 hours after the first exchange of the medium, the experiments were started by adding the pretreated water samples to final concentrations of 100, 10, 1 and 0,1% to the cultures (3 dishes for SCE- and chromosomal aberration analysis and 1 dish for the micronuclei assay), thereafter the cultures were incubated

for 3 h. Untreated cultures served as controls. The medium was then removed, the dishes washed twice with fresh medium to completely remove any mutagen/carcinogen, and fresh medium, supplemented with EGF (40 ng/ml) and BrdU (10 μM) was added.

Cytogenetic analysis

Cytogenetic studies were performed in principle as described (1). After treatment the cultures were returned to the incubator and covered with an aluminium foil to shield them from light. 48 h later colcemid (0,4 μg/ml) was added and the cultures incubated for further 3 h (the appearance of the hepatocyte cultures is documented in Fig. 1).

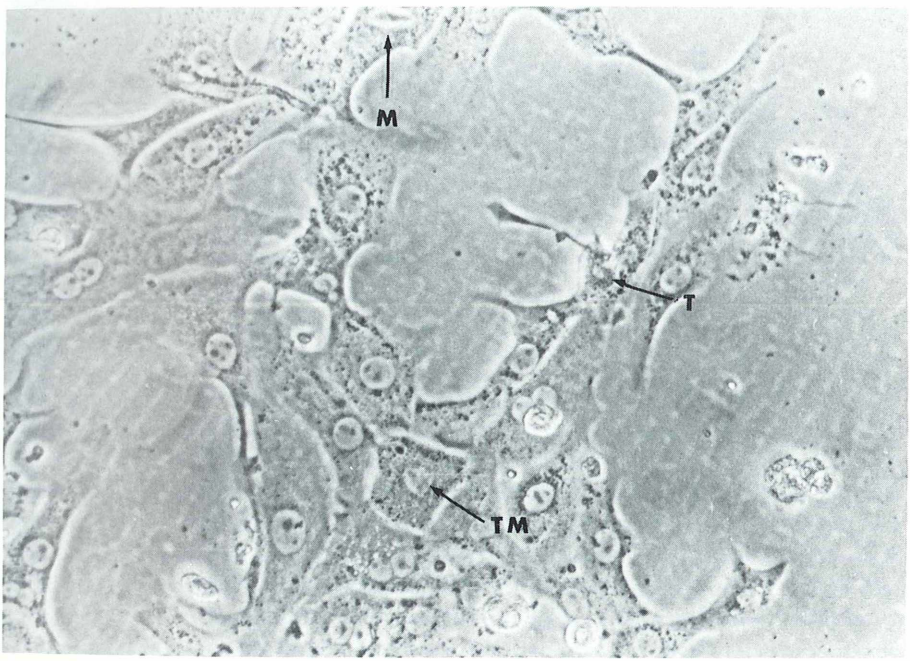

Fig. 1: Primary culture of adult rat hepatocytes at the third day of cultivation; T = telophase, M = metaphase, TM = tripolar metaphase

For SCE- (an example is shown in Fig. 2) and chromosomal aberration analysis, the medium was replaced with 2 ml of collagenase solution (0,5 mg/ml), and the plates returned to the incubator for additional 10 min. The detached cells were collected by centrifugation, treated with hypotonic solution (0,01 M KC1) for 10 min and fixed in cold methanol-glacial acetic acid overnight. Preparations were made by dropping the cell suspension in fixative on precleaned glass slides followed by flame drying.

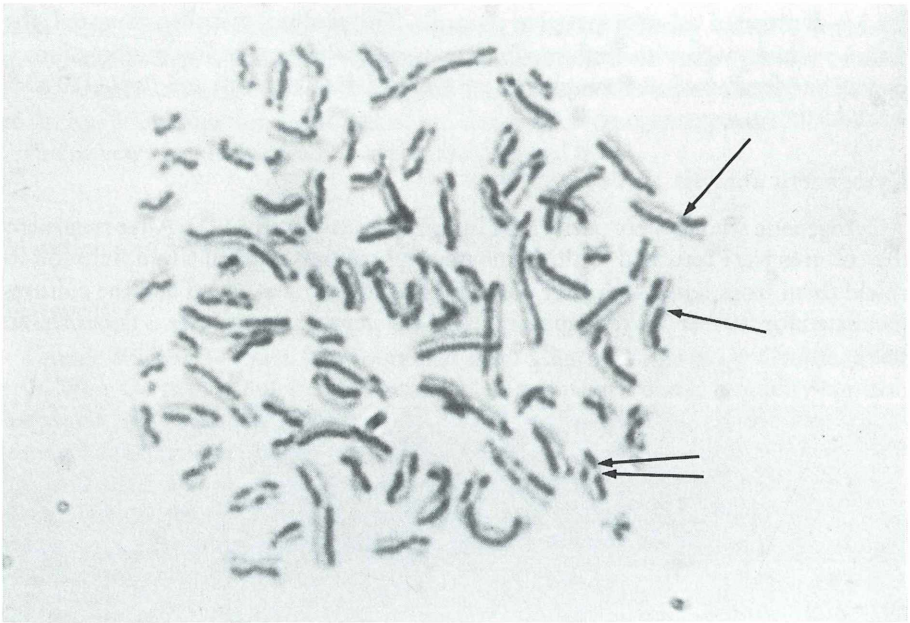

Fig. 2: Differentially stained hepatocyte metaphase (4n) exhibiting sister chromatid exchange (arrows)

The slides were stained with Hoechst 33258 (4,5 μg/ml) for 15 min, rinsed with distilled water, mounted with a coverglass in PBS (pH=7) and exposed for 1 h to a 40-W blacklight lamp (Philips TLD 36W08) on a warming plate kept at 50 °C. After removal of the coverglasses the slides were briefly washed in distilled water and stained in 5% Giemsa solution. For determining chromosomal aberrations and SCE 20 well spread metaphases – except for concentrations yielding a very low rate of 2nd division metaphases – for each parameter were scored individually to obtain the mean ± SD for a single experiment. The number of aberrations is given per diploid cell (a liver cell population is composed of diploid, tetraploid and octoploid cells, mono- or binucleated), i.e. 42 chromosomes. The SCEs are reported per chromosome.

For the determination of the mitotic index and the number of micronuclei, the cells were fixed with cold methanol-glacial acetic acid for 5 min in the petridishes, rinsed with distilled water for 2 min and airdried. The fixed cells were stained with DAPI (0,2 μg/ml McIlvaine citric acid – Na_2HPO_4 buffer, pH=7) for 30 min, washed with McIlvaine buffer for 2 min, briefly rinsed with distilled water and mounted in glycerol. To determine the mitotic index and the number of cells with micronuclei 1000 cells were analyzed under the fluorescence microscope (Reichert Univar).

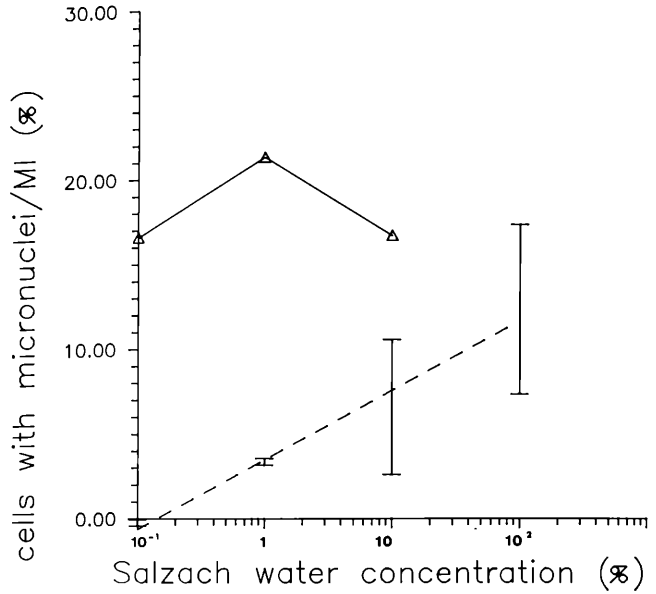

Fig. 3: Dose response of micronuclei induction by a Salzach water sample taken near the Ueberfuhrsteg in Salzburg city. The solid line represents the results of the experiment carried out the day after sampling, whereas the dashed line represents the best fit curve for three additional experiments carried out >14 days after sampling

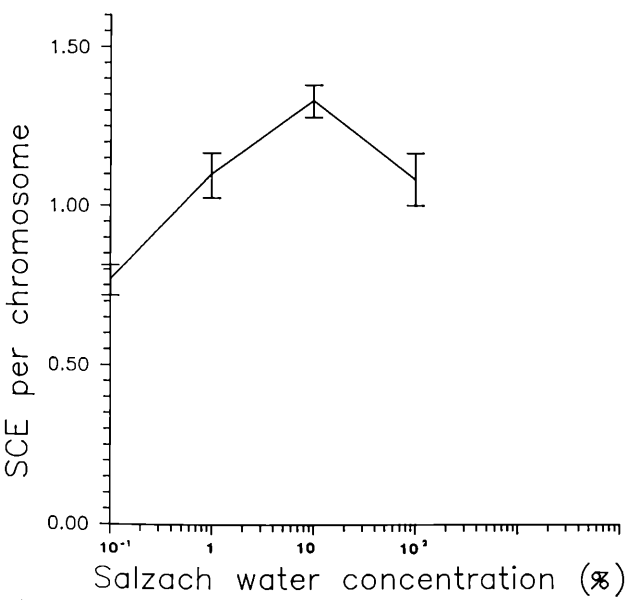

Fig. 4: SCE induction by the Salzach water sample taken near the Überfuhrsteg. The data were obtained from the experiments performed > 14 days after sampling. In the first experiment no 2nd division metaphases could be detected

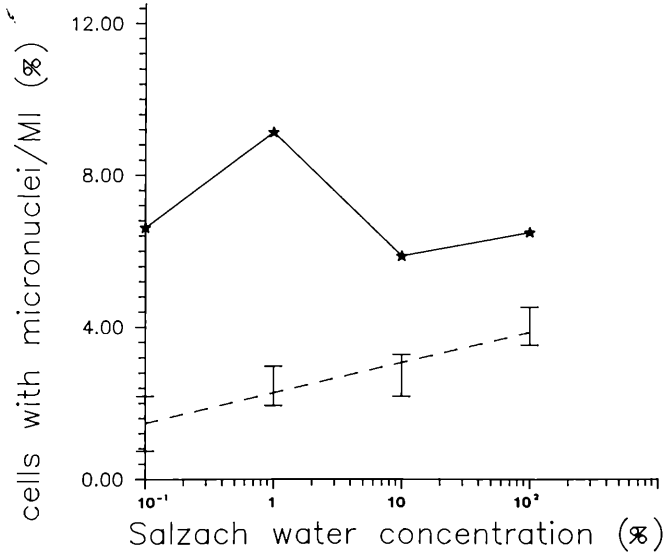

Fig. 5: Dose response of micronuclei induction by a Salzach water sample taken close to the hydroelectric power plant Urstein. The solid line represents the results of the experiment carried out the day after sampling, whereas the dashed line represents the best fit curve for three additional experiments carried out >14 days after sampling.

Results

To determine the suitability of primary cultures of rat hepatocytes for the analysis of potential mutagenic/carcinogenic properties of waters, selected samples from the river Salzach were taken in the most contaminated section (water quality IV – heavily contaminated water with little or no dissolved oxygen, 9). The results obtained are illustrated in Figs. 3–5.

Fig. 3 shows the rate of micronuclei induction of a water sample taken near the Überfuhrsteg in Salzburg. Four independent experiments were carried out for each sample at different times after sampling. The first experiment was performed the day after sampling (★) and induced the highest level of micronuclei. Even at a concentration of 0,1% highly elevated levels were observed.

The dashed line in Fig. 3 represents the regression curve for 3 additional experiments carried out more than 2 weeks after sampling. It clearly demonstrates that the genotoxic effect of the water sample dramatically decreased. However, even at 0,1% significantly elevated ($P<0,1$) levels of micronuclei are still found. With higher doses the mutagenic effect increases.

For the first experiment no SCE data could be obtained due to the toxic effect of the water-sample. SCE analysis requires differentially stained 2nd division metaphases, which were not present in this experiment. Together with the decrease of mutagenicity also the toxicity of the sample decreased so that the next experiments yielded a sufficient amount of differentially stained metaphases. The SCE data

obtained are illustrated in Fig. 4. Compared to micronuclei induction SCE show a different dose response: no significant increase of SCE at 0,1% ($P > 0,5$), a significant increase up to 10% ($P < 0,001$) followed by a decrease at 100% ($P < 0,02$).

Compared to this sample the sample taken near the hydroelectric power plant Urstein showed an approximately two times higher rate of micronuclei induction in the experiment performed the day after sampling (Fig. 5). The nondiluted sample (100%) was found to be toxic. Although the residual mutagenicity after 14 days was still higher than in the sample from Überfuhrsteg, there was also higher variation between the individual experiments. Statistically significant differences are thus only given for 1 ($P < 0,1$) and 100% ($P < 0,05$).

Whereas almost no chromosomal aberrations were induced by sample 1, sample 2 induced a slightly increasing number of mainly chromosome-, but also chromatid-type aberrations. The highest level was found for 10%. At 100% the rate of chromosome aberrations is reduced.

Discussion

The preliminary results of this study demonstrate that primary cultures of adult rat hepatocytes are very sensitive indicators for the contamination of surface waters with mutagens/carcinogens. As also shown for selected mutagens (1,10) the most sensitive cytogenetic endpoint is sister chromatid exchange induction followed by micronuclei-induction. The least sensitive is the induction of chromosome- and chomatid-type aberations.

Remarkable is the fact that the dose-response of SCE induction shows an optimum at 10% and a significantly ($P < 0,01$) reduced SCE-level at 100%. Normally the dose response is either linear with a certain slope, or saturation-type curves are obtained. Nevertheless there could be a rather simple explanation for the dose response curve obtained in this study: With increasing doses of any genotoxic agent DNA replication and cell division will be reduced at a certain rate. This means that the number of readable 2^{nd} division metaphases will approximate zero. The few cells entering a second round of cell division must also be less damaged, or must have the damage better repaired otherwise they would also stick somewhere between the first and second division. In other words the reduced rate at the highest dose could be due to the emergence of a more resistent subpopulation which could either be hepatocytes or fibroblasts. Fibroblasts are known to have a limited capacity to metabolize chemical mutagens/carcinogens. Togehter with the fact that they have a very low background SCE-level the dose dependent increase of SCEs would be much lower, so that once this cell type comes up, less SCE would be found. In fact, however, the contamination with fibroblasts is very low and was found to be $0,066 \pm 0,08\%$ after 72 hours in culture (1). Therefore it can be assumed that the decline of SCEs might be due to a hepatocyte subpopulation.

Another interesting observation was the decrease of mutagenicity of the samples with the time of storage. Obviously a large part of the initial mutagenicity is due to compounds which readily decay or which are volatile. Nevertheless there is a

remaining stable fraction of mutagens/carcinogens. This is indicated by relatively low standard errors of the pooled experiments carried out >14 days after collecting (Especially for sample 1, Fig. 3). In a next step these mutagenic compounds will be analyzed.

Surface water and ground water are interdependent which means that there is exchange of material between these water complexes. It therefore can be assumed that the mutagenic compounds present in the water samples analyzed are able to enter the ground water and to contaminate the drinking water which is taken from ground water wells. For this reason we plan to analyze ground water in the vicinity of the river Salzach especially in the highly contaminated section between Hallein and Oberndorf.

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