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Quantitative Analysis of Pollutant-induced Lethal and Sublethal Damage in Cultured Mammalian Cells

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There is a growing need for methodology to assess the potential biological toxicity of newly developed chemicals being introduced into the environment. It would be most desirable if the propensity of such chemicals to induce adverse biological effects, particularly in humans, could be easily and quickly assessed before they are developed on a commercial scale. Animal experiments, while of unequivocal predictive value, are expensive and time-consuming, and therefore not very practical as a basis for rapid and routine screening of chemicals for biological toxicity. By contrast, in vitro studies with mammalian cells in culture are much less expensive, easy and rapid to execute, and of considerable predictive value. For example, the single cell plating techniques developed by Puck and Marcus (1955) have made possible highly quantitative measurements of the effects of radiation on cell survival (ELKIND & WHITMORE 1967). The present study demonstrates that single cell plating techniques can also be used to quantify the lethal and sublethal effects of toxicants on mammalian cells and to examine the kinetics of their toxic action. Two toxicants -- pentachlorophenol and p-nitrophenol -- have been used to illustrate the efficacy of the procedures.

MATERIALS AND METHODS

The cells used in this study are a sub-line (V79-S171-W1) derived from the V79-S-171 line of Chinese hamster fibroblasts. This sub-line has been used previously for a number of studies in which the effects of radiation as well as cold and freezing damage on cell survival have been determined by assaying colony formation (KRUUV et al. 1972; RAAPHORST & KRUUV 1977; LAW et al. 1979). The cells were maintained at 37°C on plastic tissue culture dishes in Eagle's basal medium supplemented with antibiotics, L-glutamine and 15% fetal calf serum. In some experiments the fetal calf serum supplement was replaced by a mixture of 25% fetal calf serum and 75% donor calf serum with no perceptible effect on plating efficiency. The culture dishes were maintained in a humid atmosphere of 95% air - 5% CO_2, and the final pH of the medium in the incubator was 7.4. The cells grew with a doubling time of about 12 h and were maintained in the exponential phase by subculturing twice per week.

For survival curve and fluorescence depolarization experiments, cells were harvested from monolayer cultures by treatment with 0.25% trypsin - 0.1% disodium ethylenediaminetetracetate

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(EDTA) in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (CMS). The dissociated cells were then suspended in growth medium at a concentration of 5×10^4 cells/ml, and a known number were plated on 60-mm plastic petri dishes containing 5 ml of medium. For the untreated controls, only 100 cells were plated to ensure easy counting of colonies, but for the treatments, larger numbers of cells were plated in order to obtain a statistically valid number of colonies in the face of high killing rates. After 3 h at 37°C, the medium was aspirated, and the control plates received fresh medium and the treatment plates received medium containing either pentachlorophenol (99+% high purity grade from Aldrich Chemical Co. Inc.) or p-nitrophenol (99% pure from Baker Chemical Co.) at specified concentrations. The plates were placed back in the incubator and at the end of the treatment period the medium was again aspirated and replaced with fresh medium. After 7-10 days at 37°C, the medium was removed and the resulting colonies were stained with methylene blue for 45 min, rinsed with water and counted as described by Puck and Marcus (1955). Cells to be used for fluorescence depolarization measurements were harvested after 1 day at 37°C by treatment with 0.25% trypsin-0.1% EDTA in CMS, labelled with 10^{-6}M 1,6-diphenyl-1,3,5-hexatriene (DPH) as described previously (BOROCHOV & BOROCHOV 1979), centrifuged at 200g for 10 min and resuspended in phosphate buffered saline (PBS) at a concentration of 5×10^5 to 1×10^6 cells/ml. Fluorescence depolarization measurements were made with an SLM Model 8000 spectrofluorometer, and the results were expressed as anisotropy parameter $[(r_o/r)-1]^{-1}$, where r_o and r are the limiting (0.362 for DPH) and measured fluorescence anisotropy, respectively (SHINITZKY & BARENHOLZ 1978).

Pentachlorophenol and p-nitrophenol were added to the growth medium as ethanol solutions such that the final concentration of ethanol in the medium was 0.5%. The survival results were all normalized to the plating efficiency, which was determined by plating untreated cells in medium containing 0.5% ethanol. Solubility limits of the two chemicals in growth medium containing 0.5% ethanol were determined spectrophotometrically. The chemicals were added in known amounts until no further increase in UV absorbance was apparent at the wavelengths of maximum absorbance for each chemical. The quantity of medium was then doubled, and the UV absorbance of the diluted chemical again determined. From calibration curves of UV absorbance versus mg/ℓ of chemical, any further dissolution of chemical present in the original solution could be measured and thus the solubility limit in the growth medium accurately determined.

RESULTS AND DISCUSSION

Pentachlorophenol and p-nitrophenol have low solubilities in aqueous systems, and it proved necessary to add 0.5% ethanol to the growth medium in order to facilitate their solubilization. However, no significant lethal effect of ethanol was discernible up to a concentration of 1%. The solubility limits of pentachlorophenol and p-nitrophenol in growth medium containing 0.5% ethanol were 120 and 150 ppm respectively.

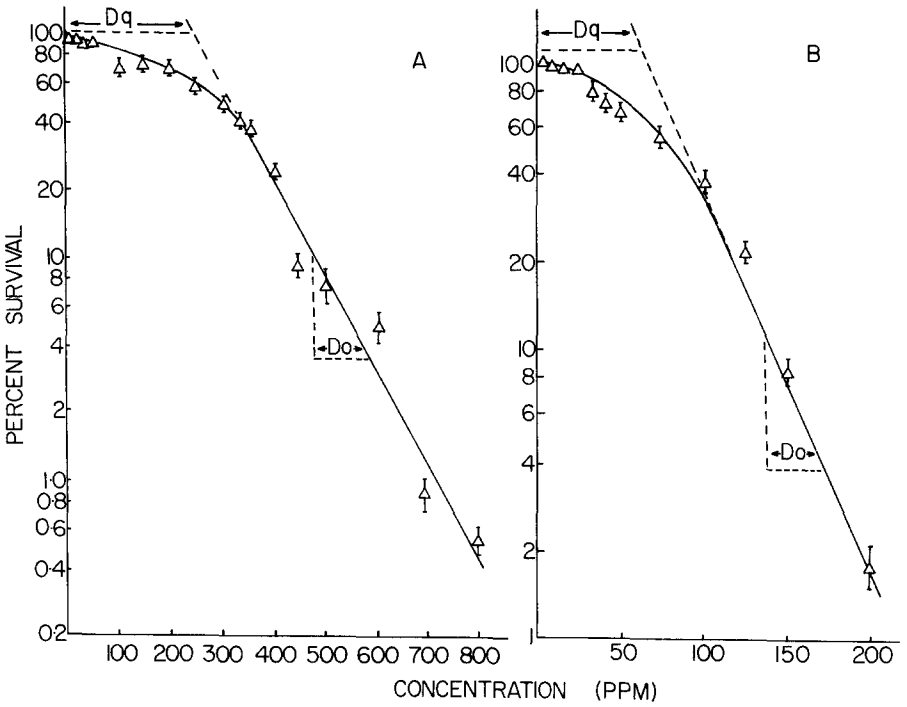

Figure 1. Survival curves illustrating the dose-response relationships for V-79 cells treated with p-nitrophenol (A) and pentachlorophenol (B). Treatments were for 24 h. Standard errors of the means for separate experiments are indicated when larger than the symbol; n=8-14. Dq is defined as the dose over which sublethal damage can be accumulated; Do is related to the killing sensitivity and is the reciprocal of the slope of the straight line portion of the curve.

For survival curves illustrating the response of V-79 cells to varying doses of pentachlorophenol and p-nitrophenol, individual cells were plated before treatment, and thus survival was scored as the retention of reproductive integrity (Fig. 1). The curves are characterized by an initial shoulder at lower doses reflecting accumulation of sublethal damage, followed by a straight portion as the dose is increased, which depicts exponential cell killing. Two quantitative parameters -- Do and Dq, which have been formulated from radiation survival curves (ELKIND

& WHITMORE 1967), can be used to describe these curves. Do is determined from the straight portion of the dose-survival curve and is defined as the dose required to reduce the number of clonogenic cells to 37% of the starting number (Fig. 1). It is the reciprocal of the slope of the straight portion of the survival curve and can thus be construed as a measure of the sensitivity of the cells to killing by the toxicants (i.e. the smaller the value of Do, the greater the killing per unit dose). Dq has been termed the quasi-threshold dose (ELKIND & WHITMORE 1967) and is defined as the dose at which the straight portion of the survival curve, extrapolated backward, intersects the dose axis drawn through 100% survival (Fig. 1). Thus, Dq is in effect the width of the initial shoulder on the survival curve and serves as a measure of the dose over which sublethal damage can be accumulated before becoming lethal.

Values for these parameters were markedly different for pentachlorophenol and p-nitrophenol (Table 1). The quasi-threshold dose (Dq) for p-nitrophenol was approximately 4-fold higher than that for pentachlorophenol, indicating that the cells are more tolerant to higher concentrations of p-nitrophenol than of pentachlorophenol. Similarly, the Do value for pentachlorophenol was about 1/3 of that for p-nitrophenol, signifying that at lethal doses the cells are about three times more sensitive to pentachlorophenol than p-nitrophenol. This difference in killing sensitivity is also apparent from LC_{50} values calculated by probit analyses (S.A.S. Institute, 1979) of the survival curve data (Table 1).

TABLE 1

Quantitative parameters describing the effects of treatments with pentachlorophenol and p-nitrophenol on the survival of V-79 cells.

Treatment	Do	Dq	LC_{50}	95% Fiducial Limits for LC_{50} values	
				Lower	Upper
Pentachlorophenol	33 ppm	64 ppm	95 ppm	87.1	104.3
p-nitrophenol	100 ppm	250 ppm	278 ppm	250.4	313.4

The application of single cell plating techniques to environmental toxicology is of particular importance because they discriminate between lethal and sublethal damage. The parameter Dq is in effect a quantitative measure of the dose over which sublethal damage can be accumulated before becoming lethal. The markedly

different Dq values for p-nitrophenol and pentachlorophenol attest to the usefulness of this parameter as a comparative measure of the extent to which toxicants can induce sublethal damage. The parameter Do is also of considerable quantitative value, for it provides a comparative measure of the relative killing efficacy of the toxicant. Judging from this parameter and from LC_{50} values, pentachlorophenol is about three times more toxic than p-nitrophenol, an observation that is in reasonable accord with oral-rat LD_{50} values of 50 mg/kg for pentachlorophenol and 350 mg/kg for p-nitrophenol reported previously (Registry of Toxic Effects of Chemical Substances, 1977).

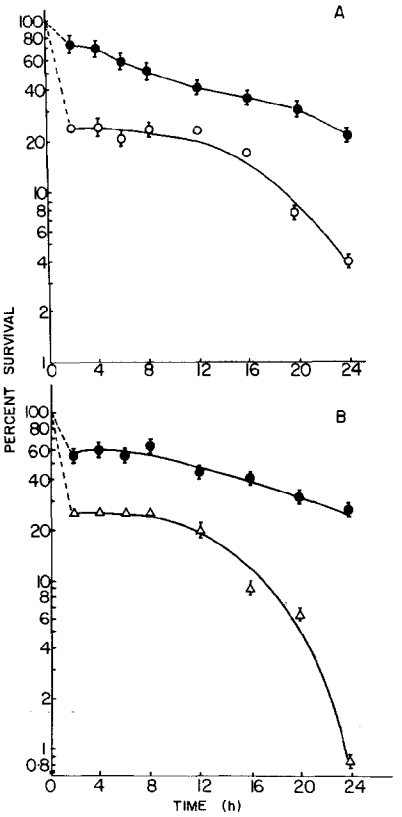

Figure 2. Survival curves illustrating the kinetics of pentachlorophenol and p-nitrophenol toxicity to V-79 cells. (A) Pentachlorophenol at 100 ppm (•) and 150 ppm (o). (B) p-Nitrophenol at 400 ppm (•) and 700 ppm (o). Standard errors of the means for replicates within experiments are indicated when larger than the symbols; n=3.

In another series of experiments, the kinetics of cell killing in the presence of pentachlorophenol and p-nitrophenol were examined. Cells were treated with different concentrations of pollutant for intervals ranging from 2 to 24 h. For pentachlorophenol, the degree of cell killing increased as a function of exposure time at each of two concentrations tested:- 100 ppm and 150

ppm. However, the kinetic curve for 150 ppm pentachlorophenol showed distinct changes in rates of cell killing with time (Fig. 2A). For p-nitrophenol, the degree of cell killing was also enhanced with exposure time, and at the higher concentration tested there were again changes in the rate of cell killing (Fig. 2B). In addition, for both pentachlorophenol and p-nitrophenol there was an immediate drop in survival at the smallest exposure time tested (2 h). This response is atypical of that depicted in classical kinetic survival curves, which normally feature a shoulder over the very early exposure times reflecting accumulation of sublethal damage. The seemingly precipitous drop in survival within 2 h of exposure to pentachlorophenol and p-nitrophenol (Fig. 2) may be due to the fact that these pollutants are lipid soluble and not readily washed out of the cell membranes when the treatment-medium is replaced with fresh medium prior to incubation of the plates for colony formation.

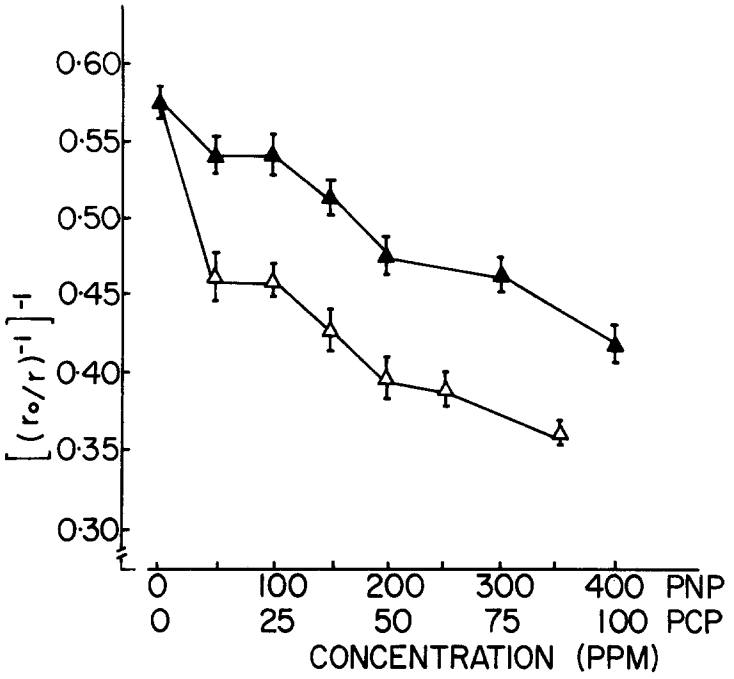

Figure 3. Changes in anisotropy parameter $[(r_o/r)-1]^{-1}$ for V-79 cells labelled with diphenylhexatriene after a 24 h treatment with various concentrations of p-nitrophenol (▲) or pentachlorophenol (Δ). Standard errors of the means are indicated; n=3 or 4. PCP, pentachlorophenol; PNP, p-nitrophenol.

Fluorescence depolarization measurements indicated that cells sustaining sublethal damage as a result of treatment with pentachlorophenol or p-nitrophenol also had increased membrane fluidity, which was quantified as a decrease in anisotropy parameter $[(r_o/r) -1]^{-1}$ after labelling with DPH (Fig. 3). Cells were treated for 24 h with concentrations of pentachlorophenol and p-nitrophenol causing sublethal damage, and then washed free of pollutant and left in culture for an additional 24 h before being harvested and labelled with DPH for fluorescence depolarization measurements. Values for the anisotropy parameter decreased by about 20% for pentachlorophenol and 35% for p-nitrophenol as concentrations of the toxicants were increased over the dose ranges causing sublethal damage (Fig. 3). Inasmuch as DPH partitions primarily into the plasma membrane when added to intact cells (BOROCHOV et al 1976), these changes in anisotropy parameter largely reflect perturbation of the plasmalemma incurred as the toxicants partition into the lipid bilayer and intercalate between the lipid molecules. However, the prospect that some internal cytoplasmic membranes were also labelled with DPH is not precluded.

P-nitrophenol and pentachlorophenol were used at concentrations above their solubility limits in the culturing medium (120 and 150 ppm respectively) in all of these experiments. Despite this, however, a graded killing response was obtained for each of the chemicals even up to concentrations (200 ppm for pentachlorophenol and 800 ppm for p-nitrophenol) far in excess of their solubility limits. This may well reflect partitioning of the pollutants into cell lipids, particularly membrane lipid bilayers (PACKHAM et al. 1980, Fig. 3), for their strongly hydrophobic nature suggests that they could accumulate in such environments to much higher concentrations than could be solubilized in the culturing medium. The kinetics of cell killing revealed that the cells become more sensitized to both pollutants with time of exposure, and this may also reflect a time-dependent accumulation of the compounds into the hydrophobic regions of membranes.

The traditional approach to obtaining predictive data in environmental toxicology has been to determine such parameters as the LD_{50} and LC_{50} from studies with whole animals. The present study has demonstrated that *in vitro* single cell plating techniques with mammalian cells in culture can provide detailed quantitative data that distinguish between lethal and sublethal damage, and provide a measure of killing sensitivity and innate biological tolerance. Inasmuch as these techniques are relatively inexpensive to deploy and generate data quickly, they could conceivably be used to generate standardized data of considerable predictive value for a range of different environmental toxicants.

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