

Application of Small Lipid Nanoparticles in *In Vitro* Mutation Assays

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ABSTRACT

We illustrate here the application of small lipid nanoparticles in genotoxicity testing using the human lymphoblastoid TK6 assay. The lipid nanoparticles were vesicles formed by lecithin encapsulating an oil core whose genotoxicity was to be evaluated. We show that the toxicities of nanoparticles were reflective of their lipid composition. Nanoparticles containing an isopropylmyristate oil core were found to be cytotoxic when present at 2.5% vol/vol in the medium. Those based on sunflower oil and triglycerides were not cytotoxic to TK6 cells when present at up to 5% vol/vol in the medium. These particles can also serve as convenient vehicles for oils and lipid soluble compounds. To check the efficiency of this delivery route, we compared the responses of TK6 cells treated with benzo(α)pyrene dissolved in DMSO or encapsulated in nanoparticles. The mutagenic responses observed in cells treated with benzo(α)pyrene solubilized in sunflower oil and delivered to the cells as nanoparticles were similar to those in cells treated with benzo(α)pyrene in DMSO. Nanoparticles were thus found to be efficient in delivering mutagens to cells in an *in vitro* mutation assay.

INTRODUCTION

In vitro mutation assays based on bacteria and mammalian cells are widely used to determine the genotoxicity of different substances. Efficient protocols are available to test purified compounds that are soluble in solvents such as ethanol or dimethyl sulfoxide (DMSO) (reviewed by Aaron et al., 1994). The method of choice to check either lipophilic compounds that are not easily soluble, or mixtures present in contaminated oils, is not obvious. The mode of delivery of oils in mammalian cell mutation assays to check for their genotoxicity has been

a problem. Currently, different solvent extracts of the oils or suspensions of oils in different emulsifiers are used (Petrilli et al., 1980). Both of these approaches have their limitations. The use of solvent extracts provides only a window of the genotoxic agents present in the oils. Suspensions of oils are preferred but the emulsifiers available (for example Tween 20) are cytotoxic to mammalian cells. The use of lipid nanoparticles could be one possible approach to deliver oil suspensions for genotoxicity determinations. These particles are vesicles consisting of an oil core that is encapsulated by lecithin, forming stable emulsions of oil in wa-

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ter (Mayhew et al., 1984). We will illustrate here the application of small lipid nanoparticles in genotoxicity testing using the human lymphoblastoid TK6 assay.

MATERIALS AND METHODS

Preparation of Lipid Nanoparticles

Nanoparticles were prepared by high-pressure homogenization using a microfluidizer (M-110T, Microfluids, USA) as follows (Mayhew et al., 1984). Phospholipids (Emulmetik E900, Lucas Meyer, Germany) were dissolved in ethanol and added to the water phase under stirring. In a second step, the oil phase was added. Subsequently, the mixture was passed six times through the microfluidizer at a pressure of 1200 bar. The size of the nanoparticles was determined by photon correlation spectroscopy (autosizer 2c, Malvern, United Kingdom). All nanoparticle preparations were composed of 1.2% phospholipids, 2% oil, and 15% ethanol during the preparation. At these conditions, a particle size of 100 nm in diameter could be obtained. After homogenization, the preparations were dialyzed against water and diluted to a final concentration of 0.6% phospholipids and 1% oil. These products were passed through 0.1 μm filter units (Millex-VV, Millipore, USA) to render sterile preparations suitable for cell cultures.

Cell Culture

The TK6 cells were maintained in RPMI 1640 supplemented with 2 mM glutamine and 10% horse serum (Gibco, Life Technologies, Basel, Switzerland). The cells were cultured in sterile flasks at 37°C in an incubator with 5% CO₂. The media used for experimental cultures were also supplemented with 5% gentamycin.

Cytotoxicity Assays

To determine the toxicities of the nanoparticles with the different lipid compositions, cultures of TK6 cells were treated with different concentrations of the lipid nanoparticles, both in the presence and absence of an exogenous metabolizing system (10% rat liver S9) for 4 h. Moltax arochlor1254-induced rat liver S9 was

obtained from Molecular Toxicology (Maryland, USA). Following treatment, the cells were suspended in fresh culture medium and plated at different cell concentrations in 96 well microtiter plates to determine the cytotoxicity of the treatment. After incubation at 37°C for 10 days, the number of wells showing the presence of the growth of cells was determined.

Mutation Assays

Mutation assays using the TK6 human lymphoblastoid cells were performed as described by Skopek et al. (1978) and Liber and Thilly (1982). Prior to the mutation assays, the TK6 cells were treated with CHAT medium to reduce the spontaneous mutants (Liber and Thilly, 1982). To determine the mutagenicity of benzo(α)pyrene in oil suspensions, cultures of TK6 cells were treated with different concentrations of the lipid nanoparticles prepared with 2% sunflower oil or 2% sunflower oil containing 10 mg/ml benzo(α)pyrene, in the presence of an exogenous metabolizing system (10% rat liver S9) for 4 h. Following treatment, they were maintained as stationary cultures in T-flasks with daily dilution to 5×10^5 cells/ml for 4 days or 6 days before the determination of the mutant fraction in the thymidine kinase (*tk*) locus or hypoxanthine guanine phosphoribosyltransferase (*hprt*) locus, respectively. Mutants at these loci were selected for by growing the cells in medium containing trifluorothymidine (*tk* locus) or 2-mercapto-6-aminopurine (*hprt* locus) in 96 well microtiter plates. After incubation at 37°C for 10 days, the number of wells showing the presence of the growth of cells was scored. Calculations to determine the mutant fractions were done as described by Furth et al. (1981). Chemicals were obtained from Sigma Co. (St. Louis, MO).

RESULTS AND DISCUSSION

To be useful as a delivery system of test samples for *in vitro* assays, the vehicle used should not be cytotoxic to cells. The cytotoxicities of nanoparticles containing different lipids are shown in Table 1. We found that the effects of the nanoparticles were dependent on their lipid composition. Nanoparticles containing triglyc-

TABLE 1. TOXICITY OF LIPID NANOPARTICLES ON TK6 CELLS

Concentration of nanoparticles (% v/v)	Survival of treated cells (%) ^a		
	Triglyceride	Isopropylmyristate	Sunflower seed oil
0	100	100	100
1	100	100	N.D.
2.5	100	30	N.D.
5	100	<1	100

^aSurvival was determined as described in the text and is shown relative to the nontreated control. N.D., not determined.

erides and sunflower seed oil were not found to exert any cytotoxicity when present at up to 5% of the culture volume. Nanoparticles containing isopropylmyristate were found to be cytotoxic, resulting in 30% survival at 2.5% of the culture volume and less than 1% survival when present at 5% of the culture volume.

The efficiency of nanoparticles as delivery systems for mutagen contaminated oils in human cell mutation assays was determined. Figure 1a and b show the mutagenicity of benzo(α)pyrene

dissolved in DMSO or encapsulated in sunflower seed oil at the HPRT and the TK locus of the human lymphoblastoid cells TK6, respectively. Nanoparticles containing sunflower seed oil were not found to be mutagenic under these conditions. However, nanoparticles encapsulating sunflower seed oil containing 10 mg/ml of benzo(α)pyrene were found to induce a mutagenic response when present at 2.5% of the culture volume (equivalent of 2.5 μ g/ml benzo(α)pyrene). We see from these figures that

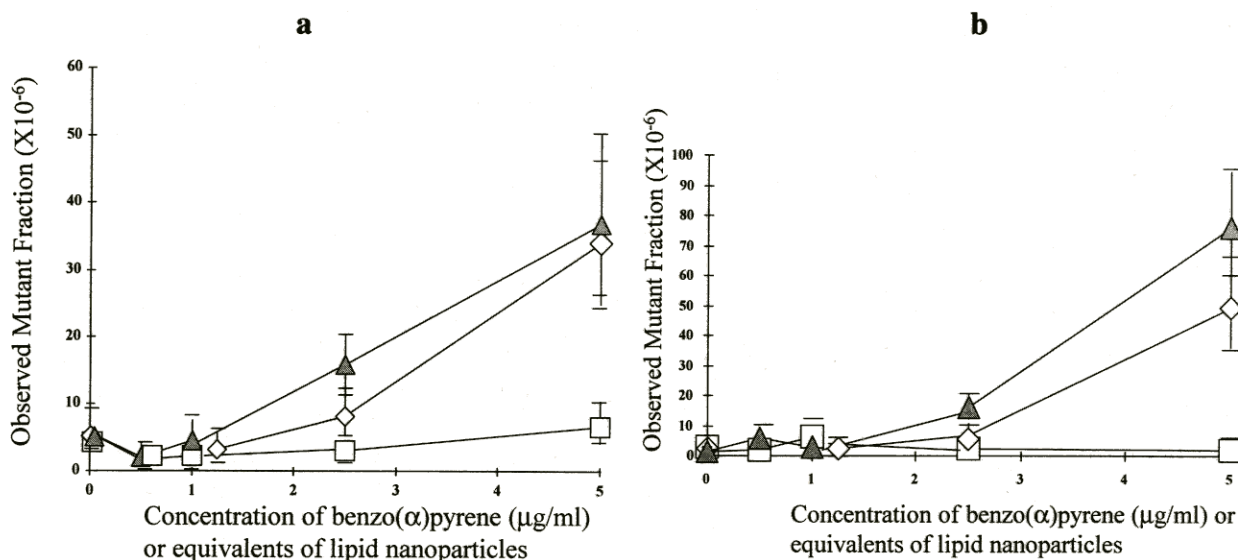


FIG. 1. Mutagenicity of benzo(α)pyrene to TK6 cells. TK6 cells were treated with benzo(α)pyrene dissolved in DMSO (\diamond), benzo(α)pyrene dissolved in sunflower seed oil and encapsulated as nanoparticles (\triangle) or sunflower seed oil (\square) in the presence of exogenous rat liver S9 extract as described in the Materials and Methods section. The observed 6TG-resistant mutant fractions (hypoxanthine-guanine phosphoribosyl transferase; panel a) and the F3TdR-resistant (thymidine kinase; panel b) are shown. The error bars show the calculated upper and lower boundary of the observed mutant fraction from two independent experiments as described in Furth et al. (1981). The concentrations shown are the concentrations of the benzo(α)pyrene added to the cultures during treatment, either in the form of benzo(α)pyrene dissolved in DMSO or dissolved in sunflower seed oil, and encapsulated as nanoparticles.

the mutagenic potencies of benzo(α)pyrene, either dissolved in DMSO or in the nanoparticle-encapsulated sunflower seed containing benzo(α)pyrene, are similar, showing that mutagens encapsulated in the nanoparticles are delivered into the cells.

The use of nanoparticle encapsulated oils is a novel method to introduce test samples in *in vitro* assays. This method can be used to test the cytotoxicities and genotoxicities of whole lipid and oil preparations. It is also particularly useful for the delivery of lipophilic compounds that are not soluble in aqueous solutions.

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