Application of flow cytometry to determine the cytotoxicity of urethane dimethacrylate in human cells

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The effects of an oligomer, urethane dimethacrylate (UDMA), on two human cell lines were studied using flow cytometry (FCM). Untreated and treated cultures of propidium iodine-stained KB (epidermal oral carcinoma cells) and human foreskin fibroblast (HFF) cells were analyzed for cellular DNA content. Concentrations of 10 and 25 μM of UDMA slightly perturbed the KB cell cycle progression at 24 and 48 h of incubation. However, the effect of 50 μM was more pronounced at the latter incubation time period. In cell growth experiments, the sublethal concentrations (10 and 25 μM) produced inhibition of KB cell growth rate at a moderate level, which resulted in the prolongation of cell population doubling time. Significant inhibition of cell growth occurred when 50 μM (lethal concentration) was used. Data obtained from the cell cycle perturbation analysis, evidenced by FCM, correlated with the extent of inhibition in KB cell growth rates. The effects of sublethal concentrations were reversible during a 24 h period of oligomer withdrawal from culture medium. In contrast, the effects of 50 μM were not reversible. In HFF cells the depletion of S phase in the cell cycle was the major effect of 50 μM of UDMA. It was concluded that FCM technology is an ideal and practical approach for studying the cytotoxicity of components of dental composites.

INTRODUCTION

With the increasing concern over the biocompatibility of synthetic materials, and a search for more reliable and reproducible *in vitro* models to measure the cytotoxicity of such materials, flow cytometry (FCM) has been used to determine cytotoxic effects of a resin component, UDMA, on cell cycle distribution. Currently, *in vitro* screening methods for testing cytotoxicity of biomedical materials, including dental materials, include morphologic effects on cell test systems, or at a functional level, effects on enzyme systems or cell membrane integrity.[1-6] Thus, the agar overlay test and the chromium release tests for membrane permeability and the molecular filter test using enzyme histochemistry for succinic dehydrogenase activity are acceptable to the International Standards Organization.[7] The agar overlay test (F895-84) and the direct contact test, which demonstrate changes in cell morphology (F895-84), are ASTM standard methods.[8] Only the chromium release assay method is listed at present as a suggested method for cytotoxicity testing by the ANSI/ADA Document 41, revision 1982.[9] These documents and standards are being revised periodically.

There are many metabolic steps that could be evaluated as measures of cytotoxic effect of various materials and devices that come in contact with living tissue. Standard tests must be specific enough to measure effects that are vital to cell function, but yet general and simple enough to be easily reproducible in a number of laboratories. The repeatability of methods such as thymidine labeling may be difficult because the method is labor intensive and the results depend largely on an understanding of the dynamics of DNA synthesis in various cell lines.[10-13,18] FCM is a method that is standardized and appropriate for materials testing. FCM methodology involves binding of dyes such as propidium iodine to DNA allowing the quantitation of DNA in each cell. In this study, FCM technology was used to study the cytotoxicity of a composite resin *in vitro,* using established human cell lines. More specifically, the cell cycle perturbations in KB and human foreskin fibroblast (HFF) cells were determined in cultures that were exposed to various concentrations of UDMA. The chemical structure of UDMA is shown in Figure 1. The incubations were 24 and 48 h in duration. To understand further the

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aberrations of the cell cycle phase assessed by FCM, the growth rates of KB cells exposed to the oligomer concentrations were also studied.

MATERIALS AND METHODS

Dental composite

UDMA was generously supplied by Esschem Company (Essington, PA). The oligomer was dissolved in 100% DMSO (Fisher Scientific, Pittsburgh, PA), and then diluted in medium so that the final concentration of DMSO was 0.1%. This concentration of DMSO had no effect on cell viability, as measured by trypan blue dye exclusion, or cell growth rates.

Cells

KB cells, an established human line derived from an epidermal oral carcinoma, were routinely grown in minimal essential medium (MEM) with Hanks' salt [MEM(H)] supplemented with 5% fetal bovine serum. Cells were passaged by conventional procedures by using 0.05% trypsin and 0.02% EDTA in a HEPES (N-2-hydroxyethoxylpiperazine-N'-2-ethanesulfonic acid)-buffered salt solution.[14] Primary HFF was grown in minimal essential medium with Earle's salts (MEM[E]) supplemented with 10% fetal bovine serum. To increase the likelihood of detecting bacterial or mixed bacterial and mycoplasma contamination, antibiotics were never used in the routine passage of both cells lines.[15] Cells were screened periodically and were mycoplasma-free.

Flow cytometry

Untreated and oligomer-treated cultures of KB and HFF cells in six-well plates were harvested and resuspended in media. Aliquots of the cells were placed into test tubes at a concentration of 3.0×10^5 cells per milliliter. Chicken erythrocytes (Whittaker Bioproducts, Walkersville, MD) were added in amounts equal to 20% of the KB or HFF cells as internal biologic standards. Samples were centrifuged at 200 $\times g$ for 5 min. The supernatants were decanted and cell pellets were resuspended in PBS, gently vortexed, and washed twice. The staining solution (PI reagent) contained 0.01 M Trisma base, 700 U/L RNase, 0.1% Nonidet P40 (NP40), 10 mM sodium chloride, and 7.5×10^{-5} M propidium iodine. One milliliter of the PI reagent was added per test tube and vortexed gently. The resulting preparations were placed in ice water and incubated for 30 min. After incubation, the nuclear suspensions were fixed by the addition of 100 μL of 2% paraformaldehyde per test tube, vortexed, and stored in the dark at 4°C overnight until flow cytometric analysis.[12]

Propidium iodine (PI) stained cells were analyzed on an EPICS 751 flow cytometer (EPICS division, Coulter Corporation, Hialeah, FL) fitted with an argon laser emitting at 488 nm (200 mW), and with a 488 nm laser-blocking filter, a long pass filter, a 560 nm dichroic filter, and a 630 nm long pass filter. Photomultiplier tube high voltage was adjusted to maintain the chick erythrocytes (CRBC) peak in channel 20. The CRBC also served as a threshold for acceptable fluorescence intensity. Events having less fluorescence than CRBC nuclei were considered debris. In all samples, a total of 10,000 nuclei were analyzed.

Data storage and analyses were performed using the Coutler EASY2 computer system. The estimated percentage of cells in G_0/G_1, S, and G_2/M phases were obtained using the PARA1 option of the EASY2 software (Coulter Electronics, Hialeah, FL), originally described by Bagwell.[17] Graphic presentation of histograms were made as described by Cameron.[16] Briefly, histogram data were translated to a file format compatible with commonly available software. For histogram comparison, the CRBC peaks were translocated to channel 20. Average DNA histograms of treated cells were generated from three separate cultures compared with three cultures of respective untreated cells.

Cell growth profile

Six-well plastic tissue culture dishes or 25 cm^2 flasks were used to study the inhibitory effect of UDMA on KB cell growth. After an incubation during which cells attached to the substrate, the medium was decanted, the cell sheet was rinsed once with HBS, and fresh medium was added. The medium consisted of MEM(H) with 0.7 mg $NaHCO_3$/L and 5% fetal bovine or calf serum plus appropriate concentrations

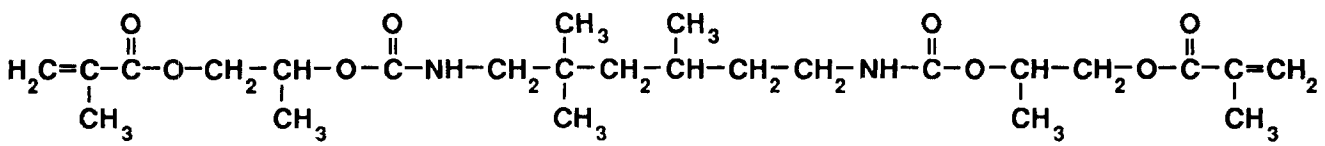

Figure 1. Chemical structure of urethane dimethacrylate (UDMA).

of UDMA. After additional periods of incubation from 24 to 72 h at 37°C, cells were harvested by means of 0.05% trypsin plus 0.02% EDTA in a HEPES-buffered salt solution, pH = 7.4. Cells were enumerated using either a Coulter Counter or a hemocytometer. Viability was determined using trypan blue dye exclusion.[2] Untreated cells throughout this study displayed 98–99% viability.

The population doubling time (PDT) for KB cells growing in the presence or absence of UDMA was subsequently calculated from the data using the subsequent equation:

$$\text{PDT} = 0.301(t)/(\log I) - (\log F) \qquad (1)$$

where I is equal to the total cell number in each well at the time when various concentrations of UDMA were added (time 0), F is equal to the average of triplicate wells after growing 48 h in the presence or absence of UDMA, and t is equal to the elapsed time (in this case, 48 h).[19]

Dose-response relationships were constructed by linearly regressing the percentage of inhibition of parameters derived in the preceding sections against oligomer concentrations. The 50% inhibitory (I_{50}) concentrations were calculated from the regression lines.[20]

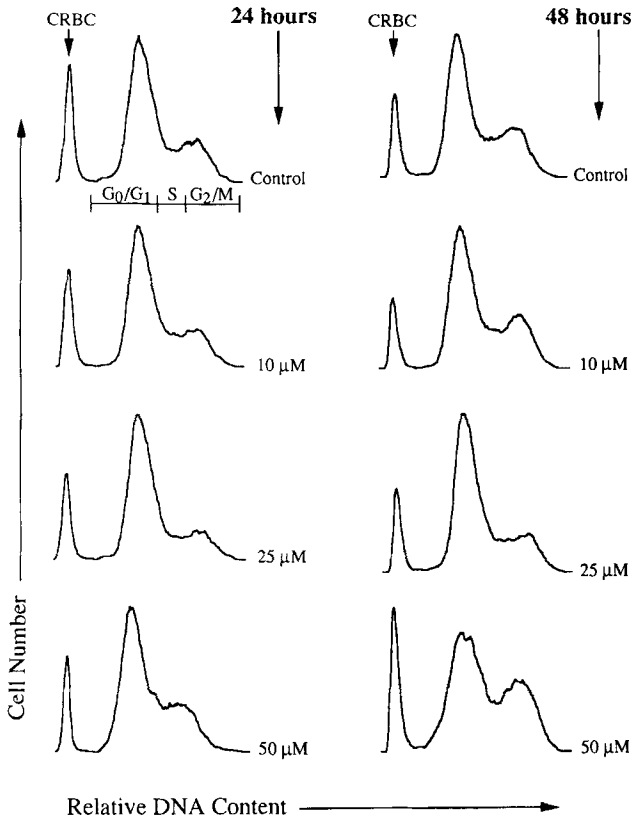

Figure 2. Distribution of DNA content frequency histogram following a kinetic response of KB cells to 10, 25, and 50 μM of UDMA after 24 and 48 h exposure times. Coefficients of variations of the G_1 peak were typically in the range of 4–5% for control cells.

RESULTS

Perturbations in cell cycle progression

The effects of UDMA on the cell cycle distribution of human cells were analyzed by FCM of PI-stained cells. The flow histograms illustrating the kinetic of cellular responses of KB cells are shown in Figure 2. Concentrations of 10 μM UDMA slightly perturbed the progression of cell cycle. A slight decrease (2%) in the population of cells in G_2/M phase was noted by 24 h of incubation. However, the effect at 48 h was more pronounced, resulting in the accumulation of cells in the S and G_2/M phases. When 25 μM was used for 24 h, the population of treated cells contained fewer cells in S and G_2/M phases when compared with untreated cells. Similar results were obtained after 48 h of incubation. The cell cycle effect at 50 μM concentration was more pronounced, resulting in significant depletion of cells from G_0/G_1 phase. In contrast, the results were different when HFF cell cultures were exposed to the same concentration of oligomer. At 24 h, more cells (11%) were accumulated throughout the G_0/G_1 phase. However, when the incubation was carried to 48 h, there was a significant depletion of cells (24%) with S phase when compared with untreated HFF cells (Fig. 3).

Inhibition of cell growth rate

Exponentially growing cultures of KB cells were treated with different concentrations of UDMA for 24 and 48 h. Figure 4 shows the effect of three concentrations of UDMA in 0.1% DMSO and medium on the number of cells per well after 48 h. At a concentration of 10 μM, the inhibitions of cell growth were 21 and 34% of control after 24 and 48 h, respectively. Cell growth suppression was significantly greater when 25 μM was used. A 63% growth inhibition was noted by 48 h. However, at shorter exposure times (24 h), the inhibition of cell growth was 52%. When 50 μM of UDMA was used, significant inhibition (91%) of cell proliferation occurred after 48 h, as compared with 74% inhibition of cell growth at 24 h. The total number of cells dramatically declined when compared with the point at which UDMA was added. Increasing concentrations of UDMA also produced a longer PDT (Table I). I_{50} concentrations were calculated for the exposure times indicated. I_{50}'s equal to 23 and 6.2 μM were obtained for 24 and 48 h of incubation, respectively (data not shown), suggesting that the growth effects were not only concentration-dependent, but also time-dependent.

Reversibility of growth inhibition

In an attempt to determine whether the suppression of KB cell proliferation seen in Figure 4 was transient, a separate experiment was performed using the identical concentrations of UDMA used in the cell growth studies. The oligomer was removed from the cell cultures after a 24 h incubation (Fig. 5). After the removal, the KB cell growth resumed for those cultures that had been exposed to 10 and 25 μM UDMA. The PDT for the recovery period for 10 μM UDMA was the same as that for the controls. For 25 μM UDMA-treated cultures, the PDT was somewhat longer during the recovery period. However, growth recovery sharply declined in cultures that had been treated with 50 μM UDMA. This observation indicates an irreversible inhibition by 50 μM UDMA, a dose that was lethal to the cells so that they were no longer capable of surviving.

DISCUSSION

Exposure of KB cell cultures to various concentrations of UDMA for two different incubation times resulted in a different set of events that led to the classification of the concentrations as sublethal (10 and 25 μM), and lethal (50 μM). As shown in Figure 2, considerable cell loss was associated with the G_1/G_0 phase at 48 h when 50 μM of UDMA was used. This loss is probably due to a retarded rate of cell entry from S into the G_2/M phase, in which the progression of cells was markedly blocked. Therefore, fewer cells recycled in the next G_0/G_1 phase (inhibition of mitosis). In a recent study, Zucker et al. identified similar cell cycle perturbation events after the exposure of murine erythroleukemic cells to methylmercury.[21] To follow up this particular finding in KB cells, experiments on the reversal of cell growth were conducted and confirmed that the cytometric aberration seen at this level of the cell cycle was a reflection of irreversible metabolic phenomena eventually leading to *in vitro* cell death. However, it is not understood whether such effects associated with the lethal concentration have biologic relevance *in vivo*. Moreover, the absence of an immune response in this *in vitro* system is another variation from the *in vivo* conditions. Certainly, animal experiments are needed to confirm the dose that results in demonstrable cell death and/or tissue necrosis *in vivo*. The mechanisms of action of UDMA and the basis of its selectivity for S and G2/M phases in KB cells, as well as its selectivity for S phase in HFF cells remains unclear. However, the changes in the frequency of distribution of DNA content at lethal dose appear to be limited to a post-DNA synthetic event. The lower concentrations of UDMA produced minor cell cycle perturbations that

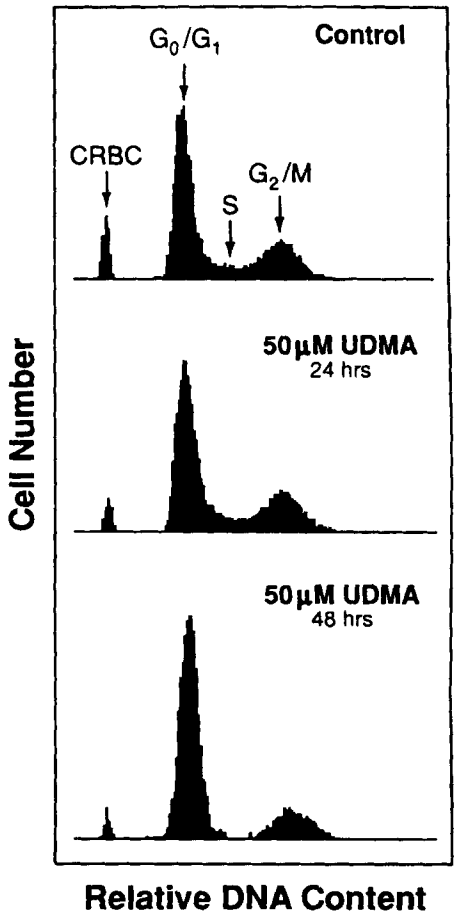

Figure 3. DNA content frequency histograms of HFF cells treated with 50 μM UDMA for 24 and 48 h. Significant depletion of the S phase cell population was observed at 48 h of incubation. Coefficients of variations of the G_1 peak were typically in the range of 3–4% for control cells.

were only cytostatic in KB cells, as confirmed by the reversal experiments. This suggests that the oligomer may affect cellular DNA polymerase rather than act as an irreversible chain terminator. The major cellular effects of the sublethal concentrations of UDMA appeared to be: 1) existence of fewer cells in G_2/M phase as compared with the untreated cultures, and 2) prolonged PDT.

After exposure of L929 cells to Doxorubicin, Lanks et al.[22] showed that there was no correlation of drug effects between DNA-FCM and a colonigenic survival assay. This suggests that there were differences in sensitivities of the colony assay and the FCM analysis. However, in the present study, there was good agreement between FCM and inhibition of KB cell growth rates. The cell cycling and cell growth effects of UDMA were dependent on both dose and time of exposure. Because of the significant cell cycle aberrations seen in the KB cells at 50 μM of the oligomer, we have further expanded this study and have used human diploid HFF cells to evaluate the

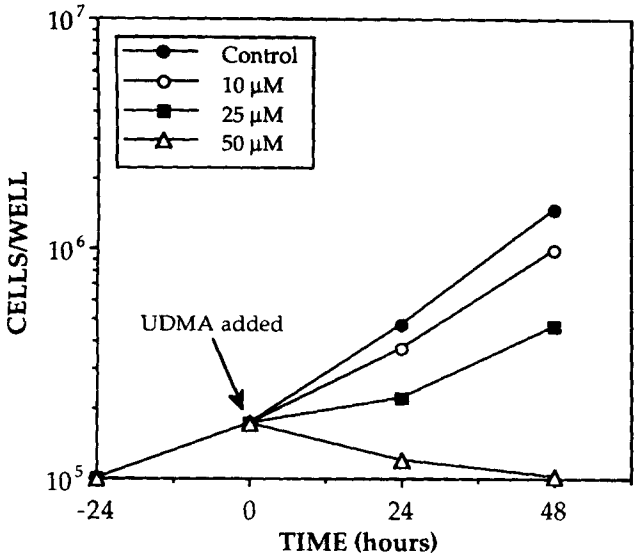

Figure 4. Effects of UDMA on growth of KB cells. Cells were seeded at 100,000 cells per well in six-well cluster dishes and incubated for approximately 24 h. At time intervals indicated, old medium was decanted and fresh medium containing selected concentrations of UDMA were added. At subsequent times, cells were harvested and enumerated with a Coulter Counter. Each data point represents the average of triplicate cultures.

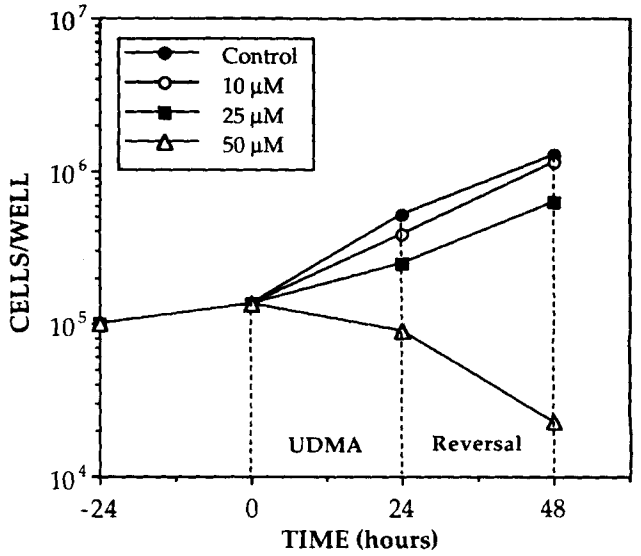

Figure 5. Reversibility of the inhibition of KB cell growth caused by UDMA. AT 24 h time intervals," various concentrations of UDMA were added and cells were incubated for another 24 h. After the second time interval, UDMA-containing medium was removed from the cells and incubation was continued with UDMA-free medium. By the end of incubation period, cells in control wells plus wells from which UDMA was removed were harvested and enumerated with a Coulter Counter. Each data point represents the average of triplicate cultures.

cell cycle effects of UDMA. The results showed that, although the progression of HFF cell cycle was also affected by 50 μM of UDMA, the phase selectivity was different from that observed in KB cells. This suggests that differential cell type sensitivity may exist when the cytotoxicity of biomaterials is tested in tissue cultures. Overall, these data indicate that the delay in cell cycle progression of both KB and HFF cells by UDMA is a function of cellular DNA integrity.

The application of FCM technology has been widely and successfully used in various fields of clinical medicine and basic science research, particularly in diagnostic pathology and cancer and antiviral research to monitor the ploidy of tumor cells, and to determine the cytotoxicity of antineoplastic or antiviral agents.[23–26] To expand further the application of this technology, it is proposed that the technology is useful in the study of the cytotoxicity of biomaterials, such as resins used in biomedical fields.

In summary, it has been shown that the oligomer, UDMA, altered the distribution of the KB and HFF cell cycle as measured by FCM, and that it functioned in both cytostatic and a cytotoxic manner in KB cell cultures, depending on the concentrations of the oligomer. The extent of cellular damage was related to the duration of exposure. A correlation emerged between the effects seen by FCM and the degree of cell inhibition. It was concluded that FCM assessment of cellular DNA content has been a substantial aid in assessing and monitoring the potentially cytotoxic responses of cultured cells to an oligomer component of resins, and that this technology offers an important tool for understanding the biocompatibility of dental and other biomedical materials.

TABLE I
Population Doubling Time (h) of KB Cells in the Presence and Absence of Increasing Concentrations of UDMA During 48 h of Exposure

Untreated	10 μM	25 μM	50 μM
16.1 ± 0.4*	19.3 ± 0.4*	34.3 ± 0.5*	> 100

*Mean ± SD. Results are the average of two separate experiments.

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