Journal of Oral Rehabilitation 2004 **31**; 1165–1172

Cytotoxic effects of dental resin liquids on primary gingival fibroblasts and periodontal ligament cells *in vitro*

Y.-L. LAI[*,†], Y.-T. CHEN[†], S.-Y. LEE[†], T.-M. SHIEH[‡] & S.-L. HUNG[‡] *[*]Dental Department, Veterans General Hospital-Taipei, [†]Faculty of Dentistry, National Yang-Ming University and [‡]Institute of Oral Biology, National Yang-Ming University, Taipei, Taiwan*

SUMMARY **Cytotoxic effects of resin liquids of three *in situ* relining dental polymers, Alike™, Kooliner, and Tokuso Rebase, and their major components, methyl methacrylate (MMA), isobutyl methacrylate (IBMA), and 1,6-hexanediol dimethacrylate (1,6-HDMA) were investigated. The concentrations of major monomers in these resin liquids were determined by high-performance liquid chromatography. Cellular viability of human gingival fibroblasts (GF) and periodontal ligament (PDL) cells were evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide assay. Moreover, patterns of cell death were analysed using annexin V/propidium iodide staining with flow cytometry. The results indicated that Alike™ liquid contained 91·3% MMA, Kooliner liquid contained 94·5% IBMA, and Tokuso Rebase liquid contained 65·8% 1,6-HDMA. All materials examined had cytotoxic effects on GF and PDL cells in dose-dependent manners. Tokuso Rebase liquid appeared to be the most cytotoxic among the various resin liquids examined. The effects of Kooliner and Tokuso Rebase liquids may have resulted from IBMA and 1,6-HDMA, respectively. Furthermore, the majority of treated cells died from necrosis; whereas a small portion of cells died from apoptosis. In conclusion, the results demonstrated that these liquid forms of dental polymers and their major monomers cause cytotoxic reactions. The direct relining procedure that cures these materials *in situ* should be used cautiously.**

KEYWORDS: **resin liquid, methyl methacrylate, isobutyl methacrylate, 1,6-hexanediol dimethacrylate, high-performance liquid chromatography, cytotoxicity**

Accepted for publication 22 October 2003

Introduction

Cytotoxic reactions to acrylic monomers such as methacrylate, the major component of most dental resin liquids, have been reported (1–9). Dahl *et al.* investigated the influence of methyl methacrylate (MMA) monomers on the cellular integrity of monocytes, granulocytes, and endothelial cells *in vitro* (3) and found that cells showed marked signs of cytotoxicity after 1 min of incubation with 10 μg mL^{-1} of MMA. Leachable substances from acrylic resins showed cytotoxic potentials in the range of their leaching concentrations (5). Eluates from various denture base resins have cytotoxic effects on oral epithelial cells (4, 6) and human gingival fibroblasts (GF) (7), as well as inhibiting cell growth, DNA replication, RNA synthesis, and metabolic processes (2, 6, 10). In addition, Cimpan *et al.* (9) demonstrated that resin eluates may enhance cell death by apoptosis and necrosis in U-937 human monoblastoid cells in a dose- and time-dependent fashion. Moreover, Tang *et al.* (8) demonstrated that direct contact of fibroblasts with methacrylate polymers, especially the unpolymerized surface layer, may more significantly reduce cell viability than what occurs with the eluates.

The direct relining technique with self-curing resins is a simple and effective method which provides a good fit for a fixed provisional prosthesis or removable denture base. Most self-curing relining resins contain MMA and/or its derivatives. Direct relining materials are usually mixed and placed *in situ* before they are polymerized. Consequently, their unpleasant odour, potential for irritation to oral tissue, and heat generation during polymerization are areas of clinical concern (11–13). To overcome these shortcomings, researchers have proposed using new acrylic monomers with increasing amounts of cross-linking agents and reducing the MMA contents of the resin liquids. Arima *et al.* (14) found that highly cross-linked acrylic relining resins had higher transverse strength and lower water sorption. Moreover, some relining materials are supposedly free of MMA and should produce less irritation to oral tissue. However, few references are available to substantiate this.

Fibroblasts constitute the predominant resident cell type of soft periodontal connective tissue which contains both gingival and periodontal ligament (PDL) fibroblasts. It is important to understand the possible effects of relining resins on these cells. In the present study, cytotoxic effects of the liquid forms of three *in situ* polymerized materials and their major components on human primary GF and PDL cells were evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Patterns of cell death induced by these materials were further analysed using annexin V/propidium iodide (PI) staining with flow cytometry.

Materials and methods

Materials

Three self-curing dental resins and three monomers were used in this study (Table 1). Alike™ is a tooth-coloured, self-curing resin for construction of temporary crowns and bridges. Kooliner and Tokuso Rebase are for hard denture linings and extension of denture borders. Based on the manufacturers' information, the major component of Alike™ liquid is MMA, while Kooliner liquid has a considerable quantity of isobutyl methacrylate (IBMA) monomer. The manufacturer claims that Tokuso Rebase liquid excludes MMA and largely consists of the cross-linking agent, 1,6-hexanediol dimethacrylate (1,6-HDMA).

Methods

High-performance liquid chromatography analysis. Concentrations of MMA, IBMA, and 1,6-HDMA in various undiluted resin liquids were determined by high-performance liquid chromatography (HPLC) as described by Larroque *et al.* with modification (15). The HPLC procedure employed a LiChroCART®250–4 HPLC cartridge and a LiChrospher® 100 RP-8 (5-μm) column*. Each solution was introduced into the injector of the HPLC system†. The HPLC was operated for 60 min until all components were completely eluted. The mobile phase was obtained with a mixture of deionized water and acetonitrile (HPLC grade) (LiChrosolv®)* at a ratio of 1:1. The flow rate was 1 mL min^{-1}. The detection wavelength was 254 nm. The HPLC traces were recorded with a standard data-capture system. Analysis of blank acetonitrile showed no interfering peaks. Concentrations of MMA, IBMA and 1,6-HDMA in the test materials were determined using a linear regression equation obtained from a calibration graph and were calculated from the areas under the curve using the EZChrom chromatography data system version 6.7‡.

Cultures of primary human gingival fibroblasts and periodontal ligament cells. Human GF were grown from explants of discarded gingival tissues obtained from medically healthy adults undergoing routine periodontal surgeries and were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% heat-inactivated foetal calf serum (FCS), 100 units mL^{-1} penicillin G sodium, 100 μg mL^{-1} streptomycin sulphate, and 0·25 μg mL^{-1} amphotericin B§ (16). The PDL tissues were scraped with a sterile scalpel blade from the midroot of extracted teeth (17). The small explants were covered with a sterile coverslip and grown in DMEM containing 10% FCS and antibiotics. Old medium was replaced with fresh medium twice a week. After migrating out of the tissue and covering the bottom of the culture plate, GF and PDL cells were detached with trypsin (0·05%)/ethylenediaminetetraacetic acid (EDTA) (0·02%)¶ and subcultured when

*Merck KGaA, Darmstadt, Germany.
†Waters Instruments, Plymouth, MN, USA.
‡Scientific Software, Pleasanton, CA, USA.
§Gibco BRL Laboratories, Grand Island, NY, USA.
¶Biological Industries, Haemek, Israel.

Table 1. Materials tested in this study

Material	Batch no.	Manufacturer	Major components
Alike™ liquid	060399	GC America Inc., Chicago, IL, USA	MMA
Kooliner liquid	041593A	GC America Inc., Chicago, IL, USA	IBMA
Tokuso Rebase liquid	044	Tokuyama Corp., Tokyo, Japan	1,6-HDMA
Methyl methacrylate	09820HI	Aldrich, Milwaukee, WI, USA	MMA
Isobutyl methacrylate	05718EU	Aldrich, Milwaukee, WI, USA	IBMA
1,6-Hexanediol dimethacrylate	366925/1 14997	Fluka, Buchs, Switzerland	1,6-HDMA

MMA, methyl methacrylate; IBMA, isobutyl methacrylate; 1,6-HDMA, 1,6-hexanediol dimethacrylate.

near confluence. Primary GF and PDL cells from passages 3 through 8 were used.

Cellular viability assay. Viability of GF and PDL cells was evaluated using MTT which measures cellular metabolic activity (18, 19). Viable GF or PDL cells (4×10^3/100 μL) were inoculated into each well of 96-well tissue culture plates and cultured for 20–24 h at 37 °C. Three resin liquids and their major components were first dissolved in dimethyl sulfoxide (DMSO) before being diluted in culture medium. The final concentration of DMSO in each sample was <0·5%. Cells were treated with various concentrations of Alike™ liquid, Kooliner liquid, Tokuso Rebase liquid, MMA, IBMA or 1,6-HDMA at 37 °C for 24 h. Cells treated with DMEM only or DMEM containing 0·5% of DMSO under similar conditions served as the controls. The cellular morphology was first examined microscopically. Unattached cells were removed by phosphate buffered saline washes. After 4 h of incubation at 37 °C with 50 μL of MTT (1 mg mL^{-1}), the supernatant was removed. Formazan crystals were dissolved in 50 μL of DMSO. After shaking, the solution was transferred to an enzyme-linked immunosorbent assay microtitre plate, and the optical density (OD) was determined at 570 nm using an EL 312e Microplate Bio Kinetics Reader**. The background signal inherent to the plates when no cells were present was subtracted from the absorbance obtained from viable cells. The OD of cells in the absence of test materials was considered to be 100%. The relative viability of GF and PDL cells was calculated by the formula: (OD of experimental sample/OD of control cells) × 100%. Experiments were performed in duplicate for at least four independent experiments, and the relative viability was averaged.

**Bio-Tek Instruments, Winooski, VT, USA.

Annexin V-FITC/propidium iodide assay. Redistribution of phosphatidylserine to the outer leaflet of the plasma membrane, which indicates the early stage of apoptosis, was detected by incubating PDL or GF with fluorescein isothiocyanate (FITC)-conjugated annexin V (Annexin V-FITC kit)††. Cells that had lost the integrity of their plasma membrane (i.e. necrotic and secondary necrotic cells) were detected by PI. Concentrations of Alike™ liquid, Kooliner liquid, Tokuso Rebase liquid, MMA, IBMA and 1,6-HDMA that affected approximately 50% of cellular viability as determined by the MTT assay were chosen. Viable GF or PDL cells (5×10^5) were inoculated into 100-mm culture dishes and cultured for 20–24 h at 37 °C. After 24 h of incubation at 37 °C with test materials, treated cells were harvested using a trypsin/EDTA solution. After being washed with 10% FCS-DMEM medium, cells were resuspended in 50 μL of 1x binding buffer and supplemented with 2·5 μL of annexin V-FITC (20 μg mL^{-1}) and 5 μL of PI (50 μg mL^{-1}) at room temperature in the dark for 15 min according to the manufacturer's instructions. After the addition of 450 μL 1x binding buffer, cells were then passed through a nylon filter (Spectrum®)‡‡. Stained cells were kept on ice and subjected to fluorescence-activated cell sorter analysis (FACS) using a Becton Dickinson FACSort flow cytometer§§ with CellQuest software§§. Green (FITC) fluorescence was collected between 515 and 545 nm, and red (PI) fluorescence was collected between 564 and 606 nm. At least 1×10^4 cells were analysed. Quadrant settings were based on the negative control. Each experiment was repeated at least three times to ensure reproducibility.

††Clontech Laboratories, Palo Alto, CA, USA.

‡‡Spectrum Laboratories, Laguna Hills, CA, USA.

§§Becton Dickinson, Oxford, UK.

Results

With calibration experiments based on five samples of known concentrations of MMA, IBMA and 1,6-HDMA, simple linear regression analysis was used to predict any unknown concentrations from the area under the peak of the chromatogram. Figure 1 shows HPLC chromatograms of three resin liquids. Retention times (t_R) of MMA, IBMA and 1,6-HDMA were approximately 6, 19 and 44 min, respectively. Alike^{TM} liquid contained 91·3% ± 0·3% of MMA. The chromatograms of Kooliner liquid (Fig. 1b) and Tokuso Rebase liquid (Fig. 1c) showed no elution of MMA. Kooliner liquid contained 94·5% ± 0·3% of IBMA. Tokuso Rebase liquid contained 65·8% ± 0·3% of the cross-linking reagent, 1,6-HDMA.

After 24 h of incubation, microscopic evaluation of oral GF and PDL cells exposed to test materials showed morphologic characteristics that did not appear normal. Pyknosis, rounding, swelling, irregular cell membrane borders and loss of attachment to the dish were evident. The possible effects of the various resin liquids, as well as MMA, IBMA, and 1,6-HDMA on the viability of primary GF and PDL cells were determined by MTT assay. This assay determines the activity of mitochondrial dehydrogenase, which is used as an indicator of cell viability. Treatment with various resin liquids impaired the viability of both GF and PDL cells in a dose-dependent manner (Fig. 2). Moreover, there was no obvious difference in the viability of GF and PDL cells. Approximately 50% of the cellular viability was affected when 0·7% of Alike^{TM} liquid, 0·2% of Kooliner liquid, 0·05% of Tokuso Rebase liquid, 1·2% of MMA, 0·15% of IBMA or 0·025% of 1,6-HDMA was used. Tokuso Rebase liquid appeared to be the most cytotoxic among the various resin liquids examined. The presence of IBMA and 1,6-HDMA explains the cytotoxic effects observed for Kooliner liquid and Tokuso Rebase liquid, respectively. However, as a higher concentration of MMA was required to obtain a similar effect on GF and PDL cells, MMA alone cannot completely explain the effects of Alike^{TM} liquid on the viability of GF and PDL cells.

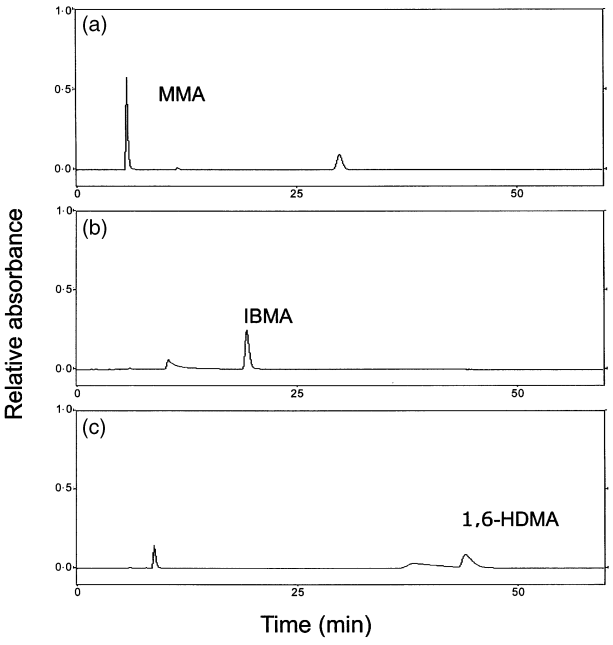

Fig. 1. High-performance liquid chromatography chromatograms of various test materials. (a) Alike^{TM} liquid, (b) Kooliner liquid and (c) Tokuso Rebase liquid. The elution of methyl methacrylate, isobutyl methacrylate, or 1,6-hexanediol dimethacrylate is indicated.

To further characterize cell death in this system, GF and PDL cells were treated for 24 h with various test materials at concentrations that affected approximate 50% of cellular viability as determined by MTT assay. Treated cells were evaluated by apoptosis analysis using FITC-conjugated annexin V and PI (20). Annexin V is a protein that binds phosphatidylserine, which is primarily localized to the inner leaflet of plasma membranes in healthy cells, but is exposed on the outer leaflet during the early stages of apoptosis (21). Propidium iodide, a DNA-binding dye, stains dead cells that have lost their membrane integrity regardless of their mechanism of death. Gingival fibroblasts were smaller and less granular compared with PDL cells as measured by flow cytometry. Representative results are shown in Fig. 3. A dot plot of green fluorescence (annexin V-FITC) versus red (PI) fluorescence shows four separate clusters: surviving cells (lower left quadrant), apoptotic cells (lower right quadrant), and secondary necrotic plus necrotic cells (upper left and right quadrants) (Fig. 3). The majority of treated cells died by necrosis; whereas a small portion of cells died by apoptosis. Apoptotic cells, which stained only with annexin V, were also visualized after treatment with MMA (Fig. 4). Similar results were obtained when cells were treated with other materials (data not shown). In addition, there were no obvious differences between GF and PDL cells.

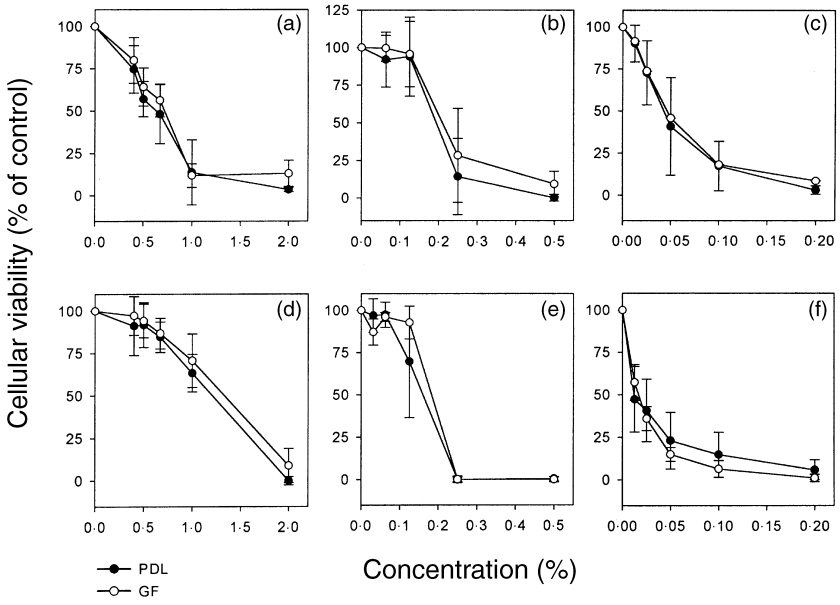

Fig. 2. Cellular viability as determined by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Gingival fibroblasts and periodontal ligament cells were incubated with increasing concentrations of (a) Alike™ liquid, (b) Kooliner liquid, (c) Tokuso Rebase liquid, (d) methyl methacrylate, (e) isobutyl methacrylate, or (f) 1,6-hexanediol dimethacrylate for 24 h, followed by incubation with MTT substrates. The results are shown as a percentage of cellular viability compared with the control reactions in which incubation was carried out in the absence of test materials. Results are the mean ± s.d. from at least four independent experiments.

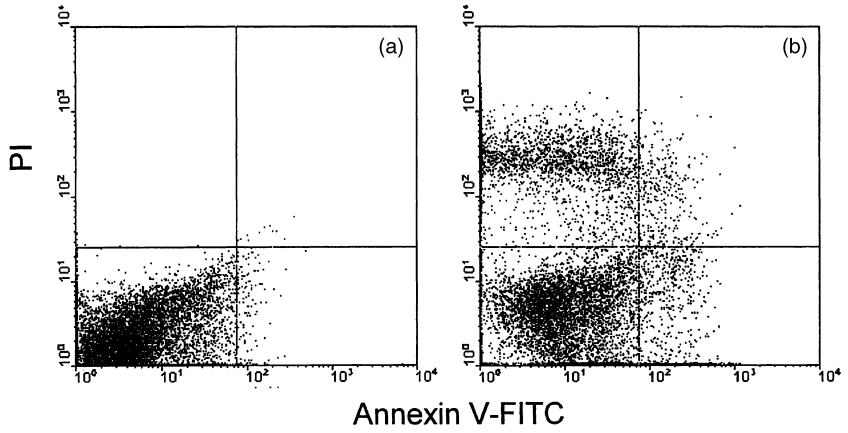

Fig. 3. Effects of methyl methacrylate (MMA) on annexin V-fluorescein isothiocyanate/propidium iodide (PI) staining of periodontal ligament (PDL) cells. (a) Untreated cells: the main group of cells is situated in the lower left quadrant (viable cells). (b) The PDL cells exposed for 24 h to MMA at a concentration of 1·2% followed by annexin V/PI staining and flow cytometry. The lower right quadrant denotes cells stained mainly by annexin V (apoptotic cells). The upper left quadrant represents cells stained mainly by PI, while the upper right quadrant represents cells stained by both PI and annexin V.

Discussion

This investigation evaluated the cytotoxicity of various relining resin liquids and their major components to primary GF and PDL cells. The three relining resin liquids chosen were a self-curing (Alike™) provisional resin liquid, and two rebasing resin liquids (Kooliner and Tokuso Rebase). Among the tested materials, the Tokuso Rebase liquid and its cross-linking agent, 1,6-HDMA, showed the greatest toxic effects, whereas MMA had the smallest effects. Both GF and PDL cells reacted similarly to the selected material and died mostly by necrosis rather than by apoptosis. Our findings clearly show that all tested materials possess certain degrees of cytotoxicity.

In this study, Tokuso Rebase liquid and its major component, 1,6-HDMA, caused severe cytotoxic damage. Yoshii (22), in a study of the relationships of monomer structures and cytotoxicity, concluded that: (i) hydroxyl groups on acrylates and methacrylates seem to enhance cytotoxicity; (ii) dimethacrylates with 23 oxyethylene chains show higher cytotoxic levels

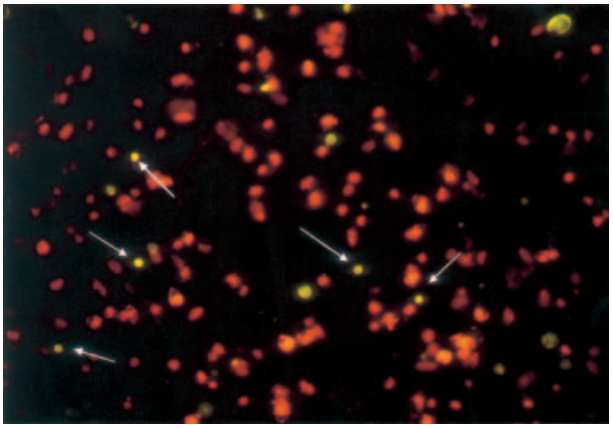

Fig. 4. Treated cells observed with a fluorescence microscope. Periodontal ligament cells were incubated with methyl methacrylate (1·2%) for 24 h followed by annexin V/propidium iodide (PI) staining. Necrotic cells were stained mainly by PI. The arrows indicate the apoptotic cells stained only with annexin V.

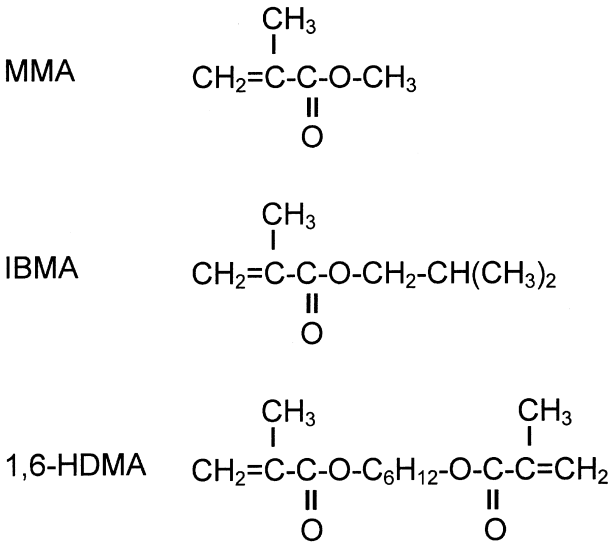

Fig. 5. Formulae of methyl methacrylate, isobutyl methacrylate and 1,6-hexanediol dimethacrylate.

than do dimethacrylates with 14 oxyethylene chains or fewer and (iii) the cytotoxic effects of monomers are correlated with the logarithm of the octanol/water partition coefficient (log *P*). Because log *P* directly relates to lipophilicity, this supports the hypothesis that the mechanism of the action of monomers is membrane-mediated and relatively non-specific (23, 24). From the longer retention time of 1,6-HDMA on the HPLC traces (Fig. 1), it could probably be inferred that 1,6-HDMA has a higher log *P*. Since Silikas and Watts (25) demonstrated a significant correlation ($r = 0{\cdot}9095$) between log *P* values and the retention time of monomers, whether 1,6-HDMA possesses higher lipophilicity (log *P*) than other materials tested remains to be examined.

Both Kooliner liquid and its major component, IBMA, showed moderate cytotoxicity. The intent of incorporating IBMA into dental polymers was to reduce water absorption by denture bases (26). However, Fujisawa *et al.* (23) revealed that the haemolytic activity and cytotoxicity of butyl methacrylate are higher than those of MMA because of its lipophilicity. This finding was supported by an investigation on the cytotoxic effects of six methacrylates with alkyl substituents on cell viability. The results indicated that the longer the alkyl chain the higher the cytotoxicity in methacrylates (22). The IBMA, which has a longer alkyl chain than MMA (Fig. 5), may have higher lipophilicity (a shorter retention time) and also demonstrated higher cytotoxicity.

A certain level of cytotoxic effects of leached MMA from cured polymer has been reported (1, 3, 5, 9, 27). The leaching period can even last for several years (28). *In vitro* studies showed that most free monomers are released into water within 1 h after polymerization (29–31). Therefore, the possible side effects of monomers are important in the first hour after polymerization (32, 33). Direct application of relining materials in the oral cavity and subsequent release of high concentrations of monomers from the initially cured resins may severely irritate the mucosa.

The mechanisms of cell death caused by relining resins have not yet been thoroughly elucidated. Two modes of cell death, i.e. necrosis and apoptosis, may be involved (34). Necrosis, referred to as cell murder, is a passive process following exposure of cells to gross injury and may trigger an inflammatory reaction in tissue (35), whereas cells that experience mild environmental stress die by the genetically controlled process known as apoptosis or programmed cell death. Apoptosis permits the organism to eliminate unwanted or subleathally damaged cells without triggering inflammatory reactions (34–36). In this study, the majority of treated cells died by necrosis; whereas a small portion of cells died by apoptosis. Cimpan *et al.* (9) also demonstrated that eluates from denture base acrylic resins might enhance cell death by apoptosis and necrosis in U-937 human monoblastoid cells. However, the proportion of U-937 cells dying by

apoptosis was higher than that in our study. This discrepancy may have resulted from the different cell cultures and resin components used in the experiments. Furthermore, induction of apoptosis and necrosis by the same chemicals may vary with different incubation times and dosages (9, 37). The detailed mechanisms necessary to initiate apoptosis by the test materials remain to be elucidated.

The GF and PDL cells are the major cellular components of periodontal soft tissues and play important roles in the maintenance of tissue integrity and regenerative processes. The precise differences between GF and PDL cells are not yet known. Our results show that primary GF cells are smaller and less granular compared with PDL cells as determined by flow cytometry analysis. Similar observations reported by previous studies suggest the presence of heterogeneous subsets of GF and PDL cells (38–41). Although permanent cell lines are recommended for cytotoxicity screening tests because of their good reproducibility, and genetic and metabolic stability (9, 42), there is concern that many cell lines may already have mutant genes which control the process of apoptosis and lead to different patterns of cell death.

Cytotoxicity as a primary factor of biocompatibility is generally determined by *in vitro* cell culture. In comparison with *in vivo* investigations, these *in vitro* studies are more easily controlled. *In vitro* methods permit assessment of various parameters in a simplified system, while minimizing variables and allowing more-specific determination of cytotoxic mechanisms. Although *in vitro* results cannot be quantitatively correlated with *in vivo* results, several clinical reports have demonstrated tissue cytotoxicity when tissue is exposed to components released from the curing resins (1, 12, 13). Oral tissue in direct contact with *in situ* polymerized resin may suffer higher concentrations of chemicals that will lead to greater tissue damage.

Acknowledgments

The authors would like to thank Dr Ying-Chieh Tsai for his advise on the HPLC analysis.

References

1. Weaver RE, Goebel WM. Reactions to acrylic resin dental prostheses. J Prosthetic Dent. 1980;43:138–142.
2. Barron DJ, Schuster GS, Caughman GB, Lefebvre CA. Biocompatibility of visible light-polymerized denture base resins. Int J Prosthodont. 1993;6:495–501.
3. Dahl OE, Garvik LJ, Lyberg T. Toxic effects of methylmethacrylate monomer on leukocytes and endothelial cells in vitro. Acta Orthop Scan. 1994;65:147–153.
4. Lefebvre CA, Knoernschild KL, Schuster GS. Cytotoxicity of eluates from light-polymerized denture base resins. J Prosthetic Dent. 1994;72:644–650.
5. Tsuchiya H, Hoshino Y, Tajima K, Takagi N. Leaching and cytotoxicity of formaldehyde and methyl methacrylate from acrylic resin denture base materials. J Prosthetic Dent. 1994;71:618–624.
6. Lefebvre CA, Schuster GS, Marr JC, Knoernschild KL. The effect of pH on the cytotoxicity of eluates from denture base resins. Int J Prosthodont. 1995;8:122–128.
7. Sheridan PJ, Koka S, Ewoldsen NO, Lefebvre CA, Lavin MT. Cytotoxicity of denture base resins. Int J Prosthodont. 1997;10:73–77.
8. Tang AT, Li J, Ekstrand J, Liu Y. Cytotoxicity tests of *in situ* polymerized resins: methodological comparisons and introduction of a tissue culture insert as a testing device. J Biomed Mater Res. 1999;45:214–222.
9. Cimpan MR, Cressey LI, Skaug N et al. Patterns of cell death induced by eluates from denture base acrylic resins in U-937 human monoblastoid cells. Eur J Oral Sci. 2000;108:59–69.
10. Schuster GS, Lefebvre CA, Dirksen TR, Knoernschild KL, Caughman GB. Relationships between denture base resin cytotoxicity and cell lipid metabolism. Int J Prosthodont. 1995;8:580–586.
11. Stenman E, Bergman M. Hypersensitivity reactions to dental materials in a referred group of patients. Scan J Dent Res. 1989;97:76–83.
12. Koda T, Tsuchiya H, Yamauchi M, Ohtani S, Takagi N, Kawano J. Leachability of denture-base acrylic resins in artificial saliva. Dent Mater. 1990;6:13–16.
13. Stanley HR. Local and systemic responses to dental composites and glass–ionomers. Adv Dent Res. 1992;6:55–64.
14. Arima T, Hamada T, McCabe JF. The effects of cross-linking agents on some properties of HEMA-based resins. J Dent Res. 1995;74:1597–1601.
15. Larroque M, Brun S. Analysis of methylmethacrylates in plasma using high-performance liquid chromatography. J Chromatogr. 1990;525:249–252.
16. Jeng JH, Lan WH, Hahn LJ, Hsieh CC, Kuo MY. Inhibition of the migration, attachment, spreading, growth and collagen synthesis of human gingival fibroblasts by arecoline, a major areca alkaloid, *in vitro*. J Oral Pathol Med. 1996;25:371–375.
17. Kapila YL, Lancero H, Johnson PW. The response of periodontal ligament cells to fibronectin. J Periodontol. 1998;69:1008–1019.
18. Mossman T. Rapid colorimetric assay for cellular growth and survival application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55–62.
19. Pellen-Mussi P, Fravalo P, Guigand M, Bonnaure-Mallet M. Evaluation of cellular proliferation on collagenous membranes. J Biomed Mater Res. 1997;36:331–336.

20. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. Anovel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods. 1995;184:39–51.
21. van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry. 1998;31:1–9.
22. Yoshii E. Cytotoxic effects of acrylates and methacrylates: relationships of monomer structures and cytotoxicity. J Biomed Mater Res. 1997;37:517–524.
23. Fujisawa S, Imai Y, Kojima K, Masuhara E. Studies on hemolytic activity of bisphenol A diglycidyl methacrylate (BIS-GMA). J Dent Res. 1978;57:98–102.
24. Dillingham EO, Lawrence WH, Autian J, Schmalz G. Acrylate and methacrylate esters: relationship of hemolytic activity and *in vivo* toxicity. J Biomed Mater Res. 1983;17:945–957.
25. Silikas N, Watts DC. High pressure liquid chromatography of dentin primers and bonding agents. Dent Mater. 2000;16:81–88.
26. Barsby MJ. A denture base resin with low water absorption. J Dent. 1992;20:240–244.
27. Dahl OE, Westvik AB, Kierulf P, Lyberg T. Effect of monomethylmethacrylate on procoagulant activities of human monocytes and umbilical vein endothelial cells *in vitro*. Thromb Res. 1994;74:377–387.
28. Sadamori S, Kotani H, Hamada T. The usage period of dentures and their residual monomer contents. J Prosthetic Dent. 1992;68:374–376.
29. Brauer GM, Termini DJ, Dickson G. Analysis of the ingredients and determination of the residual components of acrylic bone cements. J Biomed Mater Res. 1977;11:577–607.
30. Schoenfeld CM, Conard GJ, Lautenschlager EP. Monomer release from methacrylate bone cements during simulated *in vivo* polymerization. J Biomed Mater Res. 1979;13:135–147.
31. Bettencourt A, Calado A, Amaral J et al. *In vitro* release studies of methylmethacrylate liberation from acrylic cement powder. Int J Pharm. 2000;197:161–168.
32. Petty W. Methyl methacrylate concentrations in tissues adjacent to bone cement. J Biomed Mater Res. 1980;14:427–434.
33. Davy KW, Braden M. Residual monomer in acrylic polymers. Biomaterials. 1991;12:540–544.
34. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Brit J Cancer. 1972;26:239–257.
35. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol. 1995;146:3–15.
36. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell. 1997;88:347–354.
37. Demoy M, Minko T, Kopeckova P, Kopecek J. Time- and concentration-dependent apoptosis and necrosis induced by free and HPMA copolymer-bound doxorubicin in human ovarian carcinoma cells. J Control Rel. 2000;69:185–196.
38. Hassell TM, Stanek EJ III. Evidence that healthy human gingiva contains functionally heterogeneous fibroblast subpopulations. Arch Oral Biol. 1983;28:617–625.
39. Adams AM, Soames JV, Searle RF. Cultural and morphological characteristics of human periodontal ligament cells *in vitro*. Arch Oral Biol. 1993;38:657–662.
40. Hou LT, Yaeger JA. Cloning and characterization of human gingival and periodontal ligament fibroblasts. J Periodontol. 1993;64:1209–1218.
41. Kuru L, Parkar MH, Griffiths GS, Newman HN, Olsen I. Flow cytometry analysis of gingival and periodontal ligament cells. J Dent Res. 1998;77:555–564.
42. Schmalz G. Use of cell cultures for toxicity testing of dental materials – advantages and limitations. J Dent. 1994;22:S6–11.

Correspondence: Dr Shan-Ling Hung, Institute of Oral Biology, National Yang-Ming University, No. 155, Sec. 2, Li-Nong St., Pei-Tou, Taipei, Taiwan.
E-mail: slhung@ym.edu.tw