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ABSTRACT

Mechanisms by which the resin monomer 2-hydroxyethyl methacrylate (HEMA) induces hypersensitivity reactions in humans are not well-established, nor have the direct effects of HEMA on cell death been fully characterized. The objective of this study was to establish whether HEMA is capable of inducing apoptotic cell death, and whether differences exist in the levels of apoptotic death induced by HEMA in cells obtained from healthy individuals and from patients with established HEMA hypersensitivity. HEMA induced apoptotic death in Peripheral Blood Mononuclear Cells (PBMCs) obtained from both healthy and HEMA-sensitized patients and in the murine RAW cells in a dose-dependent manner. However, induction of cell death by HEMA was lower in PBMCs obtained from patients in comparison with healthy individuals. Studies reported in this paper demonstrate that HEMA induces apoptotic death, and that decreased susceptibility of lymphocytes to HEMA-mediated death might be an important mechanism for the generation and persistence of hypersensitivity reactions in patients.

KEY WORDS: apoptosis, HEMA, methacrylates, TEGDMA, CDDP, DNCB.

Resin Monomer 2-Hydroxyethyl Methacrylate (HEMA) is a Potent Inducer of Apoptotic Cell Death in Human and Mouse Cells

INTRODUCTION

The use of resin-based materials is becoming increasingly common in many fields, including dentistry. Allergic responses to such materials following skin contact have been reported in many dentists, dental assistants, and dental technicians. Clinical reports of allergic responses to resin-based restorative materials in dental personnel demonstrated that the acrylic monomer 2-hydroxyethyl methacrylate (HEMA) is the most common sensitizer responsible for the induction of severe hypersensitivity in susceptible individuals. However, with the exception of toxicity data, little is known regarding the effects of this monomer in cells and tissues. Nor is it clear how HEMA is capable of inducing hypersensitivity reactions.

There is direct evidence that released components of dental resins, and in particular HEMA and triethyleneglycol dimethacrylate (TEGDMA), do kill cells *in vitro* (Jontell *et al.*, 1995). However, there have been fewer studies on hypersensitivity responses to dental resin components in humans and in experimental animals. Induction and assessment of hypersensitivity reactions in mice have been relatively difficult until recently, when Sandberg *et al.* (2002) reported the induction of auto-antibodies against HEMA-bound self-proteins.

The cellular mechanisms which underlie the development of allergy to HEMA are not known. The present work was designed to address some of these unknowns by examining relevant cellular characteristics of immune-competent cells from the blood of humans known to be sensitized to HEMA relative to non-sensitized human controls. During the course of the work, we developed and tested the hypothesis that HEMA is a major inducer of apoptotic cell death of immune cells obtained from both humans and animals, and that HEMA is capable of mediating decreased levels of apoptotic cell death in PBMCs obtained from sensitized patients in comparison with healthy individuals.

MATERIALS & METHODS

Cells and Reagents

Recombinant IL-2 was obtained from the Chiron Corporation (Emeryville, CA, USA). The RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC). The TdT apoptosis kit was purchased from Boehringer Mannheim (Indianapolis, IN, USA). Propidium iodide, *cis*-diaminedichloroplatinum (II) (CDDP), HEMA, TEGDMA, dinitrochlorobenzene (DNCB), and RNase were purchased from Sigma (St. Louis, MO, USA). The fluorescein isothiocyanate-conjugated annexin V/propidium iodide kit was purchased from Coulter Immunotech (Miami, FL, USA). Cells were treated with IL-2 (500 u/mL), CDDP (5-20 µg/mL) HEMA, TEGDMA, and DNCB (0.082-0.00082 M) for a period of 12-18 hrs, as indicated in the RESULTS. DNCB was dissolved in DMSO before it was added to the cells.

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A supplemental appendix to this article is published electronically only at <http://www.dentalresearch.org>.

Purification of PBMCs

Identification and recruitment of patients with methacrylate hypersensitivity pose a significant limitation at present, due to physicians' and patients' lack of adequate knowledge regarding methacrylate hypersensitivity, and due to the existence of few clinical centers with the capacity to perform methacrylate patch-testing. However, despite this limitation, we were able to recruit patients with a history of hypersensitivity to HEMA, confirmed by a positive skin patch test reaction (2+ or more) to HEMA. Healthy donors were recruited and matched to our sensitized donors for age, race, and gender. All participants in this study provided informed consent, and protocols involving human subjects received UCLA Institutional Review Board approval (IRB #97-10-049-12).

PBMCs from the patients and the healthy donors were isolated as has been described previously (Jewett *et al.*, 1990, 1996, 1997; Wright *et al.*, 1998).

DNA Fragmentation

DNA fragmentation assessed by propidium iodide (PI) staining and terminal deoxyribonucleotidyl transferase (TdT) uridine triphosphate (UTP) nick-end labeling (TUNEL) assays have been described previously (Jewett *et al.*, 1996; Mori *et al.*, 1996).

FITC-Annexin V/PI Dual Staining

The staining was carried out as suggested by the manufacturer.

Statistical Analysis

Student's *t* test was performed for statistical analysis.

RESULTS

Treatment with HEMA Induced Significant Levels of Apoptotic Cell Death in PBMCs Obtained from Healthy Individuals

PBMCs from healthy non-sensitized individuals were incubated in the presence of HEMA or TEGDMA at various concentrations. A dose-dependent increase in the levels of cell death induced by HEMA was observed in PBMCs obtained from healthy individuals (Fig. 1A). Cell death occurred in both control and IL-2-treated lymphocytes. Unlike previous studies, where the induction of cell death was blocked in the presence of exogenous IL-2, the addition of this cytokine to PBMCs did not prevent HEMA-mediated cell death (Nelson *et al.*, 2003; Sarkar *et al.*, 2003). We next examined whether the induction of cell death was apoptotic or necrotic. The transferase (Tdt) uridine triphosphate (UTP) nick-end labeling (TUNEL) assay was used for the determination of DNA strand breaks representing apoptotic cell death (Overbeeke *et al.*, 1998). HEMA and DNCB, a well-known chemical inducer of hypersensitivity, caused increases in strand breaks of a similar order of magnitude, while TEGDMA induced lower levels of increase (Fig. 1B). Induction of significant apoptotic cell death by HEMA was also

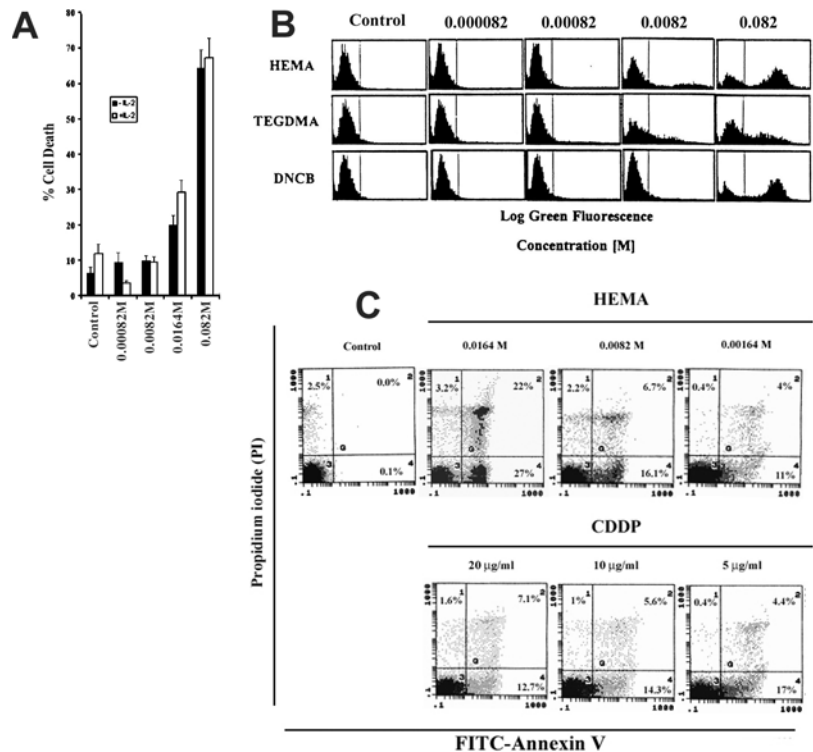


Figure 1. Induction of apoptotic cell death by HEMA. (A) HEMA at different molar concentrations was added to untreated and IL-2 (500 u/mL)-treated PBMCs (5×10^6 cells/mL) for 12-18 hrs at 37°C. Propidium iodide (PI) at a final concentration of 30 µg/mL was added to each sample, and the proportion of the PI stained cells was determined by flow cytometric analysis. The average and the standard deviation are shown for 10 separate HEMA-treated samples from healthy control donors. The two-tail unpaired *p* value for the difference between HEMA-treated and untreated samples at 0.082 M and 0.0164 M concentrations is less than 0.0001. (B) PBMCs were treated with different concentrations of HEMA, TEGDMA, and DNCB for 12-18 hrs. The levels of DNA strand breaks, indicating apoptotic cell death, were determined by the TUNEL assay. The cursor was set based on the staining obtained by control non-treated peripheral blood mononuclear cells. The events to the right of the cursor represent positive staining for apoptosis for each of the chemical treatments. One of three representative experiments is shown in this Fig. (C) PBMCs were treated with different concentrations of HEMA and CDDP for 12-18 hrs. The levels of apoptotic cell death were determined by dual staining with PI and annexin V. The numbers in each quadrant represent the percentages of cells positive for that quadrant. Ten thousand events were analyzed for each sample.

observed when dual propidium iodide and annexin V staining was performed (Fig. 1C). At the higher concentrations of HEMA treatment, more cells were observed in the early apoptotic phase when assessed by staining with FITC annexin V (Fig. 1C). Furthermore, a significant number of cells could also be seen in the later phases of apoptosis when treated with higher concentrations of HEMA (Fig. 1C). Moreover, HEMA induced apoptotic cell death in PBMCs when compared with *cis*-diaminedichloroplatinum (CDDP), a well-known inducer of cell death widely used in cancer therapy (Fig. 1C). We chose the concentrations of 5-20 µg/mL for CDDP since these concentrations were shown in the literature to mediate significant cell death in a variety of cell types (Fruhauf and Zeller, 1991; Koch *et al.*, 2003; Fischer *et al.*, 2004; Kogure *et al.*, 2004; Li *et al.*, 2004). At the highest concentration of CDDP, 80% of the cells remained both PI- and annexin-V-negative, whereas less than 50% of the cells treated with

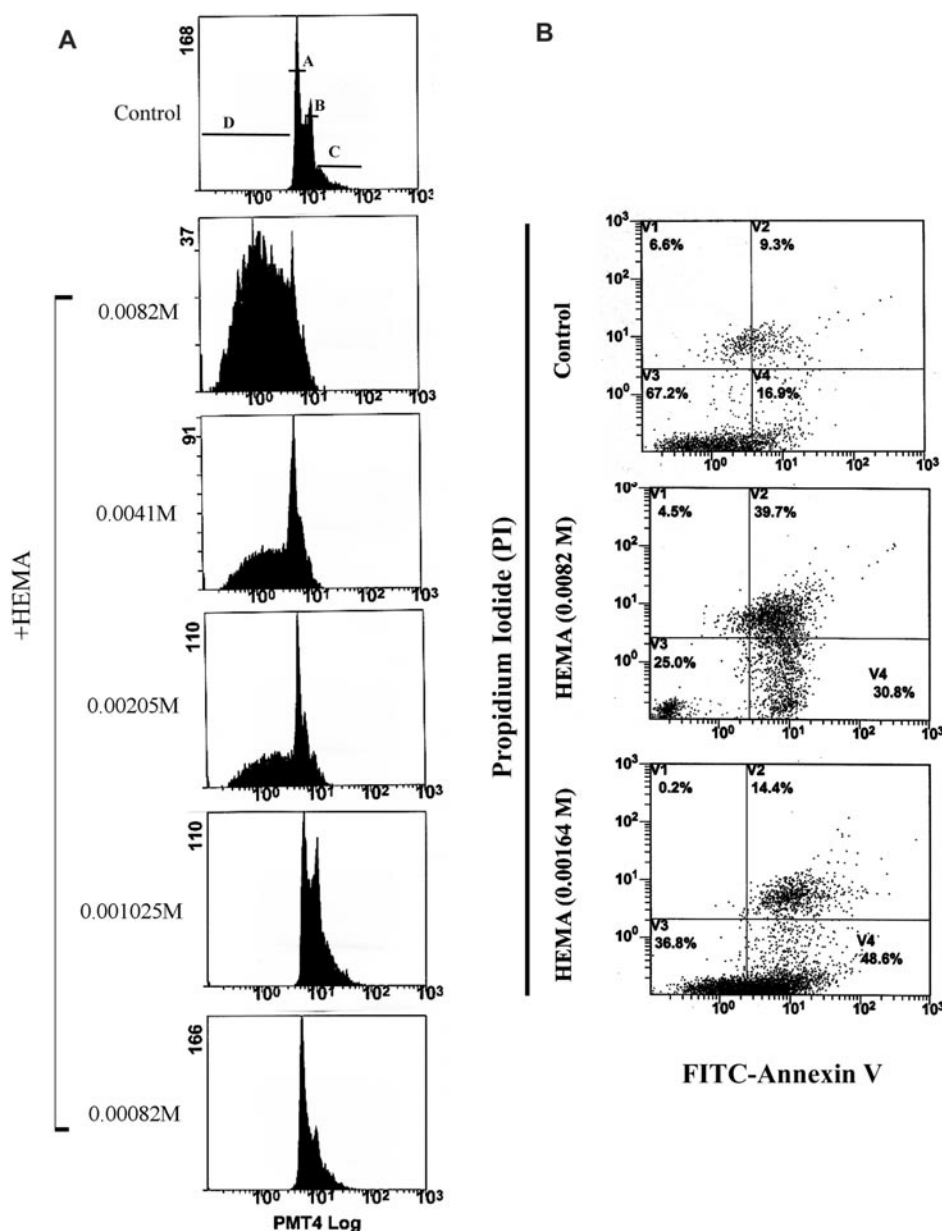


Figure 2. Induction of apoptotic cell death in mouse macrophages by HEMA. (A) Mouse macrophage cell line RAW 264.7 cells were left untreated or treated with different concentrations of HEMA (as indicated in the Fig.) in an overnight assay, and the levels of DNA fragmentation were determined by PI staining after ethanol fixation of the cells. (A) represents the proportion of the macrophage population at G_0/G_1 cell cycle, (B) represents the S phase, (C) represents the G_2/M phase, and (D) represents the sub- G_0/G_1 peak or fragmented proportion of the cells indicating apoptotic cell death. (B) Mouse macrophage cell line RAW 264.7 cells were left untreated or treated with two different concentrations of HEMA. The levels of apoptotic cell death were determined after an overnight incubation in RAW 264.7 cells dually staining with PI and annexin V. The numbers in each quadrant represent the percentages of cells positive for that quadrant. Ten thousand events were analyzed for each sample.

APPENDIX for details).

Induction of DNA Fragmentation by HEMA in Mouse Macrophages

Since HEMA was able to induce significant apoptotic cell death in a variety of cells derived from humans, we were interested to know whether this effect was specific to human cells or whether cells from murine model systems were also capable of undergoing cell death in response to HEMA treatment. Such analysis is important for the future establishment of disease model systems in mice. The mouse macrophage cell line RAW 264.7 cells were treated with different concentrations of HEMA, and the levels of apoptotic cell death were determined by propidium iodide staining of ethanol-fixed RAW 264.7 cells. Dose-dependent induction of DNA fragmentation, as evidenced by a significant increase in the sub- G_0/G_1 peak in RAW 264.7 cells, was seen in the presence of HEMA treatment (Fig. 2A). Similarly, staining with propidium iodide and annexin V indicated that the death induced by HEMA was apoptotic (Fig. 2B). Lower levels of HEMA were required for the induction of cell death in RAW 264.7 cells when compared with human PBMCs. This difference could be due to the nature of the cells used, such as normal vs. transformed cells, and/or to species differences.

Cell Death Induced by HEMA was Lower in PBMCs Obtained from HEMA-sensitized Patients Compared with Healthy Individuals

PBMCs obtained from healthy controls and HEMA-sensitized individuals were treated with different concentrations of HEMA. The levels of cell death were determined in each sample by flow cytometric analysis. HEMA induced

HEMA were unstained in the presence of PI and annexin V (Fig. 1C). A dose-dependent decrease in the levels of annexin V and PI staining could be observed when lower concentrations of HEMA were added to the cells (Fig. 1C). In addition, modulation of the genes related to cell-cycle checkpoints, signaling mediators, and apoptosis was also observed by microarray analysis, when PBMCs from healthy individuals were treated with HEMA (data not shown; see the online

lower levels of apoptotic cell death in PBMCs obtained from HEMA-sensitized patients when compared with those obtained from healthy individuals (Fig. 3A). At the HEMA concentration of 0.0164 M, 25.4% of PBMCs obtained from healthy individuals remained unstained for both PI and annexin V, whereas 64% of the cells obtained from HEMA-sensitized individuals remained unstained for both PI and annexin V (Fig. 3A). Furthermore, when analysis of forward-angle light scatter

(FLS), which represents the sizes of the cells, and side scatter (ss), representing the granularity of the cells, were considered, a significant proportion of the cell population obtained from healthy individuals had a distinct subpopulation with low FLS and high ss (Fig. 3B). Such subpopulations could not be seen in the cells obtained from sensitized patients. It is well-known that apoptotic cells initially lose their size and gain in granularity when they undergo cell death. Analysis of these data indicated that PBMCs obtained from sensitized individuals had relatively lower levels of apoptotic cell death in response to HEMA when compared with those obtained from healthy individuals.

DISCUSSION

The mechanisms by which dental resins are able to induce immune-mediated hypersensitivity reactions are not known. Using PBMCs obtained from healthy donors and methacrylate-sensitized patients, we have identified key characteristics of the immune cells, which may play important roles in the induction and persistence of chemical hypersensitivity in the patients. In this paper, we demonstrate for the first time that HEMA is a potent inducer of apoptotic cell death. Induction of cell death by methacrylates is dose-dependent, and concentrations in the range of millimole to micromole levels of HEMA were required for the induction of apoptotic cell death in PBMCs. Accordingly, we have shown, using *in vitro* analysis, that high levels (in the millimole to micromole range) of one or both of two resin monomers, TEGDMA and HEMA, were released from a range of dental materials into the adjacent aqueous phase during the first days after phase contact (Gerzina and Hume, 1994, 1995, 1996; Hamid and Hume, 1997a,b,c). Therefore, at the site where methacrylates come into contact with cellular components, local cell damage due to the induction of cell death by methacrylates may represent the initial sensitizing force. Indeed, in both the animals and human patients, we have observed large sores as a consequence of repeated application of methacrylates (unpublished observations).

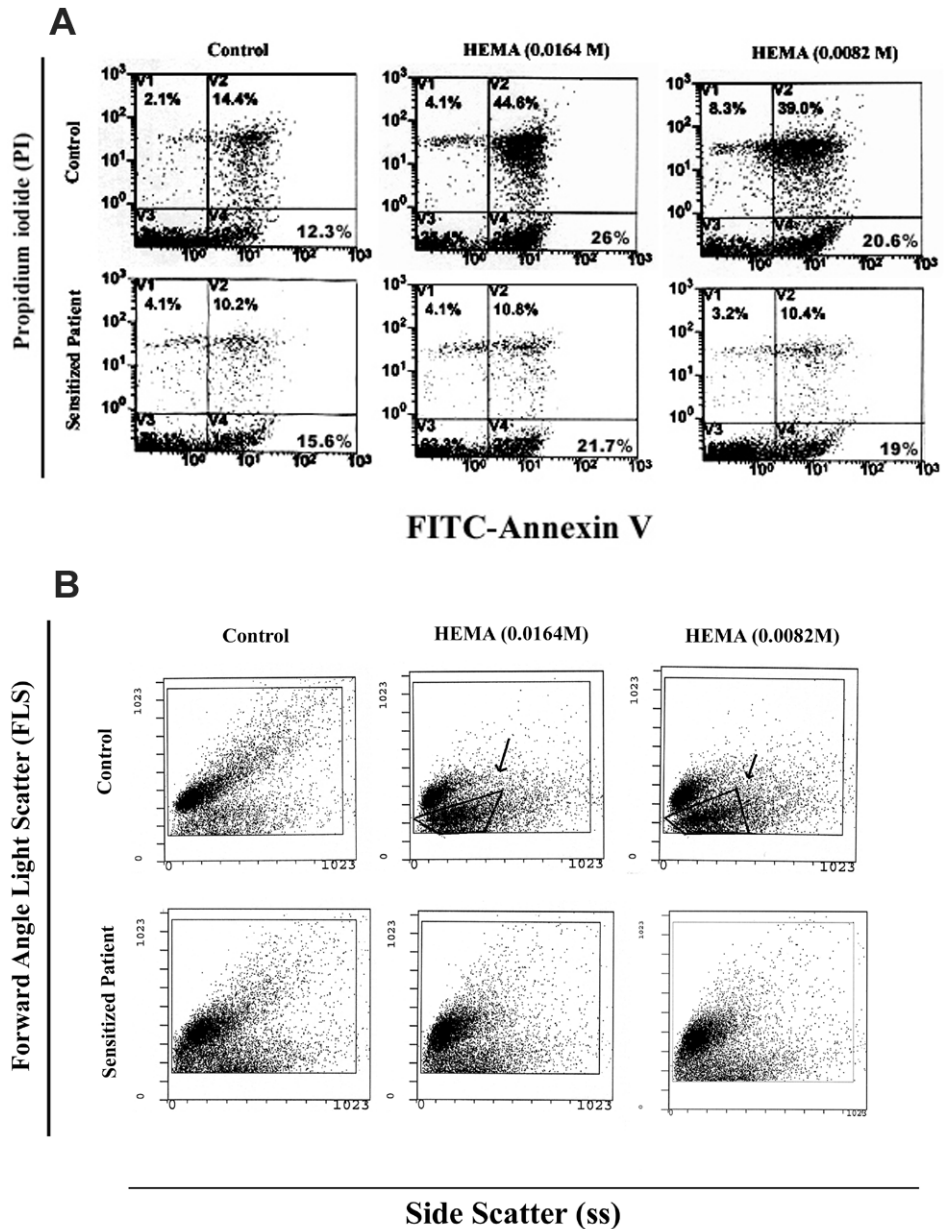


Figure 3. Decreased levels of apoptotic cell death in PBMCs obtained from HEMA-sensitized patients as compared with healthy age- and sex-matched controls. **(A)** PBMCs (5×10^6 cells/mL) from HEMA-sensitized patients and age- and sex-matched healthy controls were treated in the presence and absence of different concentrations of HEMA for 12-18 hrs at 37°C. The samples were analyzed for the levels of apoptotic cell death by dual PI/annexin V staining. The numbers in each quadrant represent the percentages of cells positive for that quadrant. Ten thousand events were analyzed for each sample. **(B)** PBMCs obtained from patient and control donors were treated with HEMA as described in Fig. 3A. The cell samples were analyzed according to two parameter of forward-angle light scatter (FLS), representing the size, and side scatter (ss), representing the granularity of the cells. Arrow is pointing to the gain in the population of cells, with decreased FLS and increased ss representing the apoptotic population in healthy control donors.

Induction of cell death was higher in PBMCs obtained from healthy individuals as compared with that in those obtained from methacrylate-sensitized patients. At the higher concentrations of HEMA, PBMCs obtained from sensitized patients underwent relatively lower levels of cell death when compared with those obtained from the healthy controls. The difference could also be observed when the levels of ^3H

thymidine incorporation and cytokine gene expression were assessed in both the healthy controls' and the HEMA-sensitized patients' PBMCs (manuscript in preparation).

Reduced sputum eosinophil apoptosis was shown to be related to increased clinical severity of chronic stable asthma (Duncan *et al.*, 2003). Likewise, the apoptosis of pulmonary lymphocytes was shown to be lower in patients with hypersensitivity pneumonitis (HP) than in normal subjects (Laflamme *et al.*, 2003). Furthermore, the persistence, severity, and duration of aspirin-hypersensitive rhinosinusitis/asthma were shown to be related inversely to the number of apoptotic immune cells (Kowalski *et al.*, 2002). These studies underscore the significance of increased apoptotic cell death in the resolution of inflammatory reaction and decreased severity of hypersensitivity reactions in patients. However, at present, it is not clear whether the decrease in apoptosis observed in HEMA-sensitized patients is specific to HEMA-mediated cell death or is a general phenomenon not specific to HEMA.

At the present time, we do not have the capability to identify and enumerate HEMA-specific memory lymphocytes. However, induction and persistence of hypersensitivity reactions in patients by dental resins may be due to the increased proliferation and expansion of HEMA-reactive memory cells in patients' blood. Alternatively, increased resistance to HEMA-induced cell death in mononuclear cells recovered from HEMA-sensitized patients may also be another reason for increased survival and proliferation of HEMA-reactive memory cells. It is also likely that a combination of both mechanisms contributes to the increased reactivity of PBMCs from methacrylate-sensitized patients. Future investigations are required to establish the mode of expansion of HEMA-reactive cells, as well as to elucidate the exact mechanisms by which HEMA-reactive immune cells may survive and mediate hypersensitivity reactions.

Analysis of the genes involved in apoptotic pathways indicated that several critical genes related to cell-cycle checkpoints, signaling proteins, and cell death mediators were elevated in the presence of HEMA treatment (see online APPENDIX). Furthermore, we have observed a decrease in the mitochondrial membrane potential and an increase in caspases 3 and 9 inductions when Jurkat T-cells were treated at the concentration of HEMA which induces significant cell death, indicating that the major effect of HEMA is likely to be mediated through the intrinsic pathway of cell death (manuscript in preparation).

There is no doubt that HEMA is a potent inducer of apoptotic cell death, since it can be shown by three distinct assay systems all specific for apoptosis. The potency of HEMA in inducing apoptotic cell death of PBMCs is comparable with that induced by DNCB, a well-known skin sensitizer commonly used to augment immune cell function in different disease states (Buckley *et al.*, 2001). However, the potency of HEMA in mediating apoptotic cell death is superior to that seen in the presence of TEGDMA. Indeed, when these monomers were added at subtoxic concentrations, the inhibitory effects of HEMA were more evident than that of TEGDMA on collagen 1, osteonectin, and DSP expression, making HEMA a more potent inhibitor of cellular function than TEGDMA (About *et al.*, 2002).

Not only may HEMA toxicity have significance in the initiation and propagation of hypersensitivity reactions, but also

it may have adverse effects on cellular functions. Even though cell death could be measured at a millimole to micromole range of concentrations, inhibition of cellular functions could be observed at much lower concentrations of HEMA (data not shown). This difference could relate to the sensitivity of the assay systems used to measure cell death vs. cellular functions. In addition, prior to the observation of significant cell death, functional inhibition of NK cell function is apparent at a much earlier time point, suggesting that, at the initiation of signaling for cell death, the inhibition of cellular function is one of the earlier events observed prior to the detection of cell death (Jewett *et al.*, 1996, 1997). Therefore, even in the case where it is difficult to obtain measurable levels of cell death in the presence of HEMA treatment, functional inhibition of the cells is a good indicator of cellular toxicity. Thus, it is not surprising to observe the inhibition of odontoblast differentiation from pulp fibroblasts at so-called 'non-cytotoxic' concentrations of HEMA and TEGDMA (About *et al.*, 2002).

In view of these results, one must not only proceed with extreme caution in using resin monomers in direct pulp-capping, but also consider the toxicity data for the selection of the least toxic material for clinical use. In addition, since these materials are highly desirable for dental restorations and esthetics, strategies should be designed to decrease or prevent toxicities mediated by unpolymerized resin materials in dental restorations, especially when polymerization is accomplished intra-orally. In this regard, our laboratory has identified candidate compounds that specifically decrease toxicities of resin monomers (manuscript in preparation).

In conclusion, we propose that HEMA mediates apoptotic cell death by inducing changes in cell-surface composition and by activating many critical genes related to apoptosis which, in turn, activate endonucleases that cause DNA fragmentation. Since it is well-known that apoptotic cells are usually taken up and cleared by antigen-presenting cells, it is tempting to hypothesize that proteins from such chemically modified cells could eventually be processed in antigen-presenting cells (APCs) and presented to T helper cells. Increased survival and activation of HEMA-specific T-cells and subsequent elaboration of cytokines could then result in the induction of allergic reactions through recruitment and activation of other inflammatory cells, such as B-cells. In addition, it has previously been shown that mammalian cells contain endogenous proteins with significant adjuvant activities which are released upon cell death (Shen and Rock, 2004). The release of such adjuvants upon HEMA-mediated cell death could represent another important step in the amplification and expansion of antigen-specific immune responses. These mechanisms are currently under investigation in our laboratory.

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