J Dent Res 74(9): 1602-1606, September, 1995

Cytotoxic Interactive Effects of Dentin Bonding Components on Mouse Fibroblasts

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Abstract. Previous studies have shown a wide range of pulpal reactions to dentin bonding systems and a poor correlation between *in vitro* and *in vivo* toxicity of dentin bonding agents. Because dentin bonding agents are composed of multiple components which may diffuse through dentin, we hypothesized that these components may cause cytotoxicity through interactive (synergistic) effects. We investigated the cytotoxicities of four dentin bonding components—HEMA, Bis-GMA, TEGDMA, and UDMA—and interactive effects for three binary combinations of the dentin bonding components—HEMA and Bis-GMA, Bis-GMA and TEGDMA, and TEGDMA and UDMA. Cytotoxicities to Balb/c 3T3 mouse fibroblasts were measured by the MTT assay. Concentrations which caused 50% toxicity compared with controls (TC_{50} values) were compared, and the interactive effects were determined by evaluation of the differences between observed and expected MTT activities of the cells. The ranks of toxicity of the dentin bonding components in terms of TC_{50} values were as follows: Bis-GMA > UDMA > TEGDMA >>> HEMA (least toxic) after 24- and 72-hour exposures. As binary combinations, the three combinations of dentin bonding components interacted in three ways—synergism, additivism, and antagonism—which were influenced by the concentrations of both components. The longer period of exposure resulted in a significant increase in the cytotoxicity of the dentin bonding components and combinations. The findings indicate that both exposure time and the interactions between the dentin bonding components may be important parameters in determining the cytotoxicity of dentin bonding agents *in vivo*.

Key words: bonding (dental), cytotoxicity, fibroblasts.

Received October 17, 1994; Accepted July 5, 1995

Introduction

Dentin bonding agents are primarily used in combination with dental resin composites to reduce microleakage of composite restorations, to provide retention with conservative preparations, and to distribute occlusal stresses (Douglas, 1989). Although clinical and *in vivo* studies have shown a low incidence of unfavorable effects of dentin bonding systems, pathological changes of pulpal tissues—such as dilatation and congestion of blood vessels, inflammatory responses, and production of irregular dentin as well as odontoblastic displacement—or tooth sensitivity can occur after placement of composite restorations (Stanley *et al.*, 1975; Ostro *et al.*, 1985; Dumsha and Beckerman, 1986; Dogon *et al.*, 1987; Chohayeb *et al.*, 1988; Bowen *et al.*, 1989; Duke *et al.*, 1991; Elbaum *et al.*, 1992). It is possible that dentin bonding agents contribute to these unfavorable pulpal reactions because they are placed directly on dentin. In addition, several lines of evidence suggest that dentin bonding agents may be a possible cause of undesirable pulpal reactions. First, Rathbun *et al.* (1991) and Hanks *et al.* (1991) showed that Bis-GMA, which is a major ingredient of most current dentin bonding systems, is easily solubilized from polymerized resins by solvents such as ethanol. Second, an oxygen-inhibited layer prevents complete polymerization of dentin bonding monomers (Rueggeberg and Margeson, 1990). Third, dentin is permeable to a variety of chemicals (Hanks *et al.*, 1994). Therefore, unpolymerized dentin bonding or resin components which remain in the cavities may diffuse to pulp *via* dentinal fluid (Pashley, 1988; Gerzina and Hume, 1995).

Since current dentin bonding agents have multiple components, interactions among these components may occur when pulpal cells are exposed, resulting in more or less cytotoxicity than the individual components would have caused by themselves. Previous *in vitro* studies of resin toxicity have not determined the interactions between the components (Dumsha and Sydiskis, 1985; Meryon and Brook, 1989; Hanks *et al.*, 1992). Available studies show that

pulpal reactions to dentin bonding systems range from none to severe. The severity of pulpal reactions may be influenced by a number of factors, such as composition, clinical application procedure, and dentin permeability (Söderholm, 1991). In the current study, we hypothesized that interactions between components of dentin bonding agents could increase the cytotoxicity of these agents relative to the individual components. The aims of this study were: (1) to assess the cytotoxicity of the dentin bonding components, HEMA, Bis-GMA, TEGDMA, and UDMA, in monolayer cell-cultures; and (2) to determine the interactive effects of toxicity for three binary combinations of dentin bonding components—HEMA and Bis-GMA, Bis-GMA and TEGDMA, and TEGDMA and UDMA—which are primarily present together in dentin bonding systems.

Materials and methods

The selected dentin bonding components and combinations for toxic assessment are major ingredients in most current dentin bonding systems (Van Meerbeek *et al.*, 1992).

Cultures of Balb/c 3T3 mouse fibroblasts, derived from clone A31 (CCL 163, ATCC, Rockville, MD), were used for evaluation of the cytotoxicity of the dentin bonding components. These cells were used because they are contact-inhibited and non-tumorigenic and thus have properties similar to those of cells *in vivo*. In addition, these cells compare favorably with primary cell lines in their cytotoxic response (Wataha *et al.*, 1994). The medium used for culture was Dulbecco's Modified Eagle's Medium (DMEM) with 4500 mg/L glucose (Gibco, Grand Island, NY), 3% Nu-Serum (Collaborative Research, Bedford, MA), and supplements (2 mmol/L glutamine, 125 units/mL penicillin, and 125 µg/mL streptomycin; Gibco, Grand Island, NY).

Balb/c 3T3 cells were plated at 8250 cells *per* well in 200 µL of medium in a 96-well dish (Falcon) and were incubated in 5% carbon dioxide, 100% relative humidity, and 37°C for 24 h before dentin bonding components were added. The final cell density was 25,000 cells/cm^2. HEMA (2-hydroxyethyl methacrylate, Batch #12012DX; Aldrich Chemical Company) was dissolved in sterile distilled water, and Bis-GMA (2,2-bis[(p-2'hydroxy-3'methacryloxy-propoxy)phenyl]propane, Batch #334-27, Esschem), TEGDMA (triethylene-glycol-dimethacrylate, Batch #334-2, Esschem), and UDMA (urethane dimethacrylate, Batch #326-28, Esschem) were dissolved in 95% ethanol. After dilution, the final concentration of 95% ethanol in the culture medium was 0.5%. One µL of various concentrations of the dentin bonding components was added to each experimental well. The controls for HEMA were 1 µL of sterile distilled water, whereas the controls for the other components were 1 µL of 95% ethanol (final concentration = 0.5%). Pilot experiments established that 1% of ethanol caused no cytotoxicity. Eight replicates were used for each concentration. After the cells were exposed to the components for 24 or 72 h, the MTT assay was used for toxicity assessment of the succinic dehydrogenase activity of the cells as well as the number of viable cells, as previously described (Wataha *et al.*, 1992). From the results, the optical densities of treated wells were compared with those of the control wells, and a dose-response curve was plotted to delineate the concentrations of the dentin bonding components which depressed MTT-formazan production by 50% (TC_{50} value). We calculated standard deviations and used ANOVA to compare TC_{50} values for the different dentin bonding components; we used a paired *t* test to compare TC_{50} values at 24 and 72 h for each component.

To assess the toxicity of the binary combinations of dentin bonding components, we plated Balb/c 3T3 cells in 96-well plates and incubated them as previously described. The concentrations of the first component were varied from plate to plate, whereas the concentrations of the second component were varied within each plate. One µL of each component was added to each well; there were 8 replicates *per* combination. Controls of the HEMA and Bis-GMA combination were 1 µL of sterile distilled water and 1 µL of 95% ethanol (final concentration = 0.5%), whereas controls of the Bis-GMA/TEGDMA and TEGDMA/UDMA combinations were 2 µL of 95% ethanol (final concentration = 1.0%). The plates were incubated for 24 or 72 h before toxicity assessment. As with single components, the cytotoxicity of the binary combinations was measured by the MTT assay. We determined the interactive effect of the dentin bonding components on the fibroblasts by calculating the difference between the observed toxicity and the expected toxicity. The observed toxicity was obtained from the experiment by analysis of the MTT activity. The expected toxicity of the specific concentrations of the combinations was calculated from the actual optical density (O.D.) of the control samples, as follows:

$$\text{Expected toxicity} = \text{O.D. (Control of Component 1)} \times \frac{\text{O.D. (Control of Component 2)}}{\text{O.D. (Control of both Components)}}$$

For example, if a concentration of component 1 caused a 40% drop in cellular activity, then a 40% drop was added to all concentrations of component 2. A three-dimensional graph for each combination was produced, with the X- and Y-axes representing the concentrations of the first and second dentin bonding components, respectively, and the Z-axis representing the difference between observed and expected values for the MTT activity. If the observed minus the expected MTT values equalled zero, it represented a simple additive effect (zero interaction). In this case, the toxicity was a simple summation of the individual effects. If the difference between the observed and the expected values was less than zero, it represented a synergistic effect, *i.e.*, the activity of the viable cells in the experiment was less than expected. If the observed minus the expected values was greater than zero, this combination had an antagonistic interaction, in which the observed MTT response was greater than expected. Confidence intervals were calculated for each point on the 3-D surface (95%).

Results

The TC_{50} values of the dentin bonding components decreased significantly at 72 h compared with 24 h (ANOVA and paired *t* test, $p < 0.001$, Figs. 1a,b). For example, the TC_{50} value of HEMA dropped from 3600 µmol/L (SD = 700) after 24 h to 1025 µmol/L (SD = 30) after 72 h. Similar decreases were observed for the other components. The rank of the TC_{50} values of the 4 dentin components was the same with either time of exposure: Bis-GMA (most toxic) > UDMA > TEGDMA >>> HEMA (least toxic).

All three types of interactions (synergistic, additive, antagonistic) occurred and were influenced by concentrations of the combinations (Figs. 2a-f). For example, at 24 h, a significant antagonistic effect was found when from 0.5 to 5 µmol/L of Bis-GMA and from 500 to 10,000 µmol/L of HEMA were present (Figs. 2a,b). Synergism of this combination was prominent when Bis-GMA was present at 25 µmol/L with any concentration of HEMA, but synergism was significant only when from 1000 to 2500 µmol/L of HEMA were present. At 72 h, there was antagonism at concentrations of 25 µmol/L Bis-GMA and all concentrations of HEMA, 750 µmol/L HEMA, and all concentrations of Bis-GMA, and 2500 µmol/L of HEMA and from 7.5 to 10 µmol/L of Bis-GMA. At 250 to 1,000 µmol/L of HEMA (except 750 µmol/L of HEMA) and 0.5 to 5 µmol/L of Bis-GMA, significant synergism occurred.

At 24 h, antagonism was more dominant than synergism and additivism for all combinations, whereas at 72 h, the occurrence of antagonism decreased and that of synergism increased considerably, especially in the combination of UDMA and TEGDMA. In addition, the HEMA/Bis-GMA and the Bis-GMA/TEGDMA combinations had similar patterns of toxic interactions at each time interval. For instance, at 24 h, 0.5 µL of Bis-GMA with any toxic concentration of HEMA or TEGDMA resulted in antagonism, whereas 10 µL of Bis-GMA with any toxic concentration of HEMA or TEGDMA resulted in synergism. This phenomenon also occurred at 72 h. However, the third combination, TEGDMA and UDMA, had a different pattern.

Discussion

There is controversy about the causes of unfavorable pulpal responses under resin composite restorations. A few studies have proposed that bacterial invasion after microleakage results in cellular pathology (Brännström and Nyborg, 1972; Bergenholtz *et al.*, 1982), whereas other reports indicate that chemical toxicity of resins could contribute to pulpal irritation (Vojinovic *et al.*, 1973; Stanley *et al.*, 1975; Franquin and Brouillet, 1988; Qvist *et al.*, 1989; Tagami *et al.*, 1990; Pashley, 1992). The present study demonstrated that, *in vitro*, synergism and antagonism between components of dentin bonding agents exist. For example, low concentrations of Bis-GMA (< 5 µmol/L) antagonized the cytotoxicity of HEMA between 8 and 20% after 24-hour exposure (Fig. 2a). On the other hand, 25 µmol/L of Bis-GMA was likely to cause synergism regardless of the concentration of HEMA, and it increased the toxicity 10% in combination with 1000 µmol/L of HEMA. Other combinations of Bis-GMA and HEMA did not show significant interaction.

The duration of exposure had a strong effect on the toxicity of dentin bonding systems, since the longer period of exposure resulted in a higher incidence of synergistic interactions. For example, the reaction of less than 5 µmol/L of Bis-GMA with HEMA resulted in an 8 to 20% toxicity reduction (antagonism) after 24-hour exposure (Fig. 2a), but after 72-hour exposure, the same concentrations increased toxicity 16% (Fig. 2b). Generally, antagonism was dominant at the 24-hour exposure time, whereas synergism became a dominant interaction at the 72-hour exposure time.

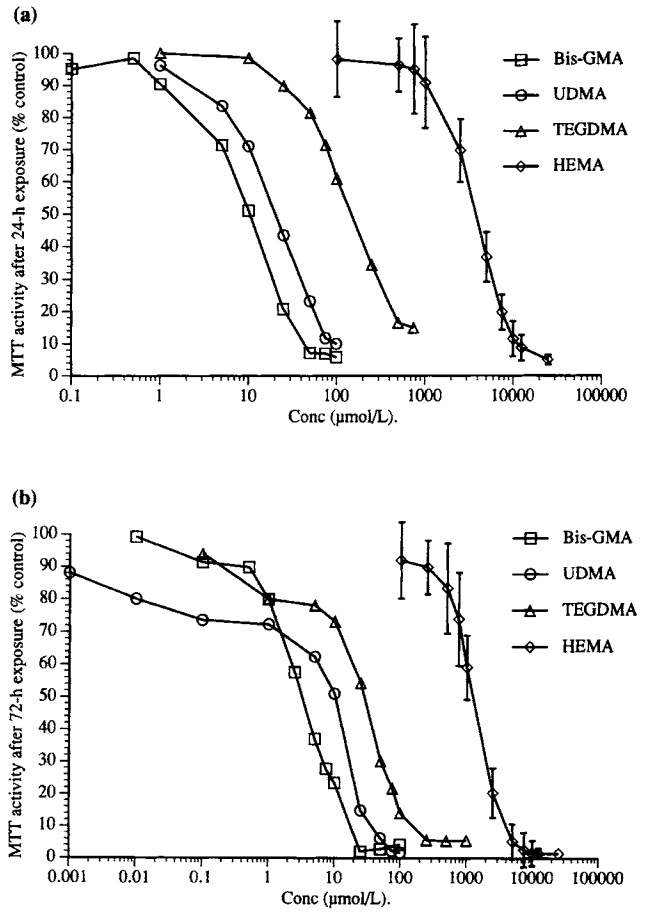

Figure 1. Cytotoxicities of the four dentin bonding components (Exp. 1). Comparison of TC_{50} values of HEMA, Bis-GMA, TEGDMA, and UDMA at (a) 24 h and (b) 72 h. At 24 h, the TC_{50} values were as follows: HEMA - 3600 µmol/L (SD = 700), Bis-GMA - 9.35 µmol/L (SD = 0.66), TEGDMA - 124.5 µmol/L (SD = 35.2), and UDMA - 17.4 µmol/L (SD = 6.16). At 72 h, the TC_{50} values were as follows: HEMA - 1025 µmol/L (SD = 30), Bis-GMA - 3.2 µmol/L (SD = 1.5), TEGDMA - 24.3 µmol/L (SD = 4.95), and UDMA - 10.01 µmol/L (SD = 0.03). Error bars (standard deviations) of each component were determined, but error bars are shown only for HEMA to reduce clutter.

Furthermore, it was observed that interactions often occurred at concentrations below those necessary for individual resins to be toxic. Therefore, when one is assessing the cytotoxic potential of a resin which releases multiple components, knowing the individual cytotoxicities of the components is not adequate. From the current study, it was clear that synergism and antagonism could exist in the same system. This may mean that multiple mechanisms are involved in the cytotoxicity of these resins—each mechanism acting at specific concentrations and time conditions. Additional evidence would be necessary to support this hypothesis.

The existence of synergistic interactions means that dentin bonding agents have the potential to cause toxic responses in pulp at lower levels than they could by themselves. Since resins are known to release concentrations which may cause these reactions, the interactions have *in vivo* relevance. For example, HEMA and TEGDMA release

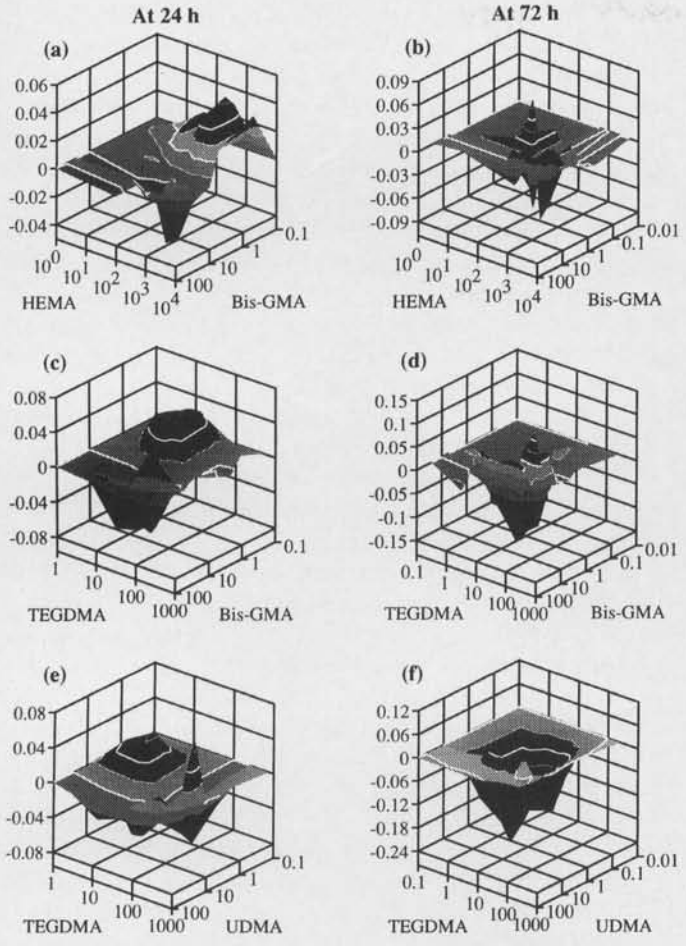

Figure 2. Interactive effects of binary combinations of dentin bonding components (Exp. 2). (a) HEMA and Bis-GMA at 24 h. (b) HEMA and Bis-GMA at 72 h. (c) Bis-GMA and TEGDMA at 24 h. (d) Bis-GMA and TEGDMA at 72 h. (e) TEGDMA and UDMA at 24 h. (f) TEGDMA and UDMA at 72 h. The X- and Y-axes represent the concentrations of the components in µmol/L, and the Z-axis represents the observed-expected MTT activity. Blackened areas of the 3-D surfaces indicate areas of effect significantly different from zero. 95% confidence intervals were 0.03, 0.03, 0.02, 0.05, 0.02, and 0.03, for (a) through (f), respectively.

at approximately 30 ± 13 and 172 ± 95 nmol, respectively, in three days (Gerzina and Hume, 1994). Rueggeberg *et al.* (1995) showed that in a copolymer of TEDGMA and Bis-GMA, Bis-GMA was the major component, and leaching was in the range of 10 to 30% of its original abundance.

The validity of the MTT assay was confirmed by comparison of the cytotoxicities of the individual components with those of previous experiments (Hanks *et al.*, 1991), which showed similar cytotoxicity results for these resins (Bis-GMA, TEGDMA, and UDMA). Cytotoxicity of HEMA in the current study was in agreement with that in another study (Bouillaguet *et al.*, 1995), but not with that in the study done by Hanks *et al.* (1992). Therefore, we verified our results by repeating the HEMA experiment several times.

Our model to determine interactions has been commonly used in pharmacology and toxicology (Magos, 1981; Prichard and Shipman, 1990). However, experimental variation was a critical factor in statistical demonstration of these interactions. Since we determined interactions by computing the observed minus expected cytotoxicities, the error of this difference was a combination of the errors for observed and expected values. We were able to minimize these errors by using eight replicates for each concentration and using the MTT assay, which provided minimal variation. We further validated our results by repeating experiments to verify the shapes of the three-dimensional surfaces.

The present study has shown that the different resins which are present in dentin bonding agents can interact to alter cytotoxicity *in vitro*. These interactions may cause the resins to be more or less toxic than the sum of the individual toxicities, but prolonged duration of exposure was likely to increase toxicity of the resins. The mechanisms of these interactions remain unknown. Since the interactions occurred at concentrations which have been shown to be released from resins *in vitro*, they may be relevant to pulpal irritation *in vivo*. However, further studies which quantify the concentrations released, the concentrations which diffuse through dentin, and the duration of such leaching are necessary for understanding of the role of these interactions in pulpal irritation.

Acknowledgments

This investigation was supported in part by USPHS Research Grant DE 09296 from the National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892.

This paper is based on a thesis submitted to the graduate faculty, University of Michigan School of Dentistry, in partial fulfillment of the requirements for the Master of Science in Restorative Dentistry.

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