Effects of an Experimental Calcium Aluminosilicate Cement on the Viability of Murine Odontoblast-like Cells

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Abstract

Introduction: Quick-setting calcium aluminosilicate cement with improved washout resistance is a potential substitute for calcium silicate cements in endodontics. This study examined the effect of an experimental calcium aluminosilicate cement (Quick-Set; Primus Consulting, Bradenton, FL) on the viability of odontoblast-like cells. Methods: The biocompatibility of Quick-Set and white ProRoot MTA (WMTA; Dentsply Tulsa Dental Specialties, Tulsa, OK) cements and their eluents was evaluated using a murine dental papilla-derived odontoblast-like cell line (MDPC-23); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to examine the effects of the 2 hydraulic cements on mitochondrial metabolic activity. Flow cytometry and confocal laser scanning microscopy were used to identify the effects of the 2 cements on cell death-induced plasma membrane permeability to fluorescent dyes and DNA stains. Results: After the first week of immersion in culture medium, Quick-Set and WMTA were more cytotoxic than the Teflon-negative control (P < .05), and the cells exhibited more apoptosis/necrosis than Teflon (P < .05). After the second week of immersion, the 2 cements were as biocompatible as Teflon (P > .05), with cells exhibiting minimal apoptosis/necrosis. Eluents from the set cements at 1:1 dilution were significantly more cytotoxic than eluents at 1:10 or 1:100 dilution (P < .05). Conclusions: Quick-Set and WMTA exhibited similar cytotoxicity profiles. They possess negligible in vitro toxicologic risks after time-dependent elution of toxic components. (J Endod 2012; 38:1–7)

Key Words
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, biocompatibility, calcium aluminosilicate, hydraulic cement, flow cytometry, MTT assay, vital cell staining

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1.8 million pulp cappings, 3.2 million pulpotomies, 23,000 apexification procedures, and 150,000 root-end filling procedures are performed annually in the United States alone (1). For the past decade, endodontists have embraced the use of calcium silicate hydraulic cements (ie, cements that set in the presence of water), such as mineral trioxide aggregate (MTA) for these procedures and for root and furcal perforations. MTA possesses excellent biocompatibility, bioactivity, and sealing properties and the ability to induce reparative hard-tissue formation. Despite these outstanding properties, there are potential disadvantages associated with the use of MTA including a long setting time, difficulty of manipulation, low resistance to washout, and potential staining of coronal dentin (2–5). Although other calcium silicate-based materials with improved handling properties have been introduced recently (eg, Biocement; Septodont, Saint-Maur-des-Fossés, France) (6), the shortcomings of MTA and MTA-like materials necessitate the development of more ideal cements for endodontic applications.

Hydraulic aluminosilicate cements for biomedical uses derived from industrial compositions are modifications of high-alumina cements used for refractory linings in furnaces and some civil engineering purposes. The aluminate cements were developed in the early 20th century to be more chemically durable than Portland cement (7). The hydraulic calcium phases typically contain 30% to 50% alumina, whereas Portland cements contain less than 5%. Calcium aluminate cements have been developed for use as bone cements (8, 9); they have strengths higher than 100 MPa, pull-out strengths equal to polymethyl methacrylate bone cement, and formed calcium alumina chlorohydrate phases. In dentistry, this type of cement has been formulated into dentin replacement restorative materials (DoxaDent; Doxa AB, Uppsala, Sweden) (10–13) and more recently as a bioactive, hybrid-glass-ionomer dental luting cement (Ceramik C&B, Doxa AB) (14–17). Calcium aluminate powder has been blended with radiopacifier powders and mixed with water or salt solution for comparisons with MTA products for the same endodontic indications (EndoBinder; Binderware, São Carlos, SP, Brazil) (18–22).

Another related calcium aluminosilicate cement (Capsio; Primus Consulting, Bradenton, FL) has been previously evaluated for its physical properties (23) and in vitro bone nodule formation by rat calvarial osteoblasts (24). Capsio is composed primarily of bismuth oxide, dental glass, and calcium aluminosilicate with a water-based gel. The material is slightly less basic (pH = 10.9) than white MTA (pH = 11.6) upon the final setting, has a setting time of 9 minutes, penetrates dentinal tubules,
and shows improved acid resistance and washout resistance versus MTA. Recently, Capasio powder has been refined and renamed as Quick-Set (Primus Consulting), and the cationic surfactant was removed from the liquid gel component, which was thought to interfere with cell cytocompatibility (24). The quick setting, fine particles, high radiopacity, and slightly elevated pH properties of Capasio were preserved in Quick-Set.

Because endodontic cements are applied in intimate contact with periradicular tissues, their biocompatibility is a prerequisite in expediting the repair of bone loss caused by host immunologic responses to microbial infections. Thus, the objective of the present study was to examine the effect of Quick-Set on the early proliferative events of murine odontoblast-like cells before their differentiation. The null hypothesis tested was that there is no difference between experimental calcium aluminosilicate cement and commercially available calcium silicate endodontic cement in affecting the viability of odontoblast-like cells.

Materials and Methods
Quick-Set and white ProRoot MTA (WMTA; Dentsply Tulsa Dental Specialties, Tulsa, OK) were mixed with the proprietary gel or deionized water, respectively, in a powder/water ratio of 3:1. The mixed materials were placed in presterilized Teflon molds (5-mm diameter and 3-mm thick) (DuPont, Wilmington, DE), covered with presterilized Mylar sheets (DuPont), and allowed to set completely in a 100% humidity chamber for 24 hours. The positive control consisted of disks prepared from Intermediate Restorative Material (IRM; Dentsply Caulk, Milford, DE), which is a zinc oxide–eugenol cement. Teflon disks of the same dimensions were used as the negative control. All set materials were sterilized with ultraviolet light for 4 hours before testing.

Cell Culture
The biocompatibility of the set cements was evaluated with an in vitro cell culture cytotoxicity model using a murine dental papilla–derived odontoblast-like cell line (MDPC-23) (25, 26). The cells were plated in a 24-well format at a density of 5,000 cells/cm² in 0.5 mL growth medium and incubated at 37°C in a humidified 5% CO₂ atmosphere for 24 hours until fully established. The growth medium consisted of Dulbecco modified Eagle medium (Lonza, Walkersville, MD) and 10% fetal bovine serum (Invitrogen Corp, Carlsbad, CA) supplemented with 2 mmol/L L-glutamine and 100 U/mL penicillin streptomycin.

Because cytotoxic components can diffuse out of set hydraulic cements into periradicular tissues, both cement disks and their eluents were evaluated using a cycling regimen developed in our laboratory (27–30). A weekly cycle consisted of direct evaluation of the toxicity of the cement disks over the plated cells for 3 days and indirect evaluation of the effect of eluents derived from the set cements on the established cells. The latter was achieved by immersion of the disks in growth medium for 4 days to collect eluents. Accordingly, during the first part of each weekly cycle, cement and control disks (n = 12) were placed individually in transwell inserts with a 3-µm pore size (BD Falcon, Franklin Lakes, NJ) to prevent direct contact of the cells by the specimen. After the inserts were placed over the plated cells, an additional 2 mL of growth medium was added to each well to ensure that the level of the culture medium was above the sides of the transwell insert. The disks were exposed to the plated cells for 3 days, without further change in culture medium, before testing for succinic dehydrogenase activity. During the second part of each weekly cycle, the disks were retrieved and incubated at 37°C with growth medium (1 disk/2 mL) for 4 days to collect the eluents (n = 12) from the set cement before using the same disks for the next cycle. For each disk, the same growth medium was used for eluent collection throughout the entire testing period. This cycling regimen was repeated weekly for 2 weeks (ie, 2 cycles) until the material disks were rendered nontoxic (ie, >90% of the mean succinic dehydrogenase activity exhibited by the Teflon-negative control) (27). Each eluent concentrate collected after the 2-week aging period was diluted with fresh growth medium to 1:1, 1:10, and 1:10² of its original concentration to achieve a final volume of 2 mL (27). Each diluted, eluent-containing growth medium was then used as the respective culture medium for freshly plated murine dental papilla–derived odontoblast-like cell line (MPDC-23) cells for testing cell viability.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyldetrazolium Bromide Assay

Cell viability was evaluated by incubating 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldetrazolium bromide (MTT) with MDPC-23 cells that had been exposed to cement disks for 3 days in a normal growth medium and an eluent-containing growth medium in various dilutions for 3 days. The cells were incubated in MTT-succinate solution for 60 minutes and fixed with Tris-formalin. The purple MTT formazan produced in the cells, as a result of mitochondrial succinic dehydrogenase activity, was dissolved in situ using dimethyl sulfoxide-NaOH, and the optical density was measured using a microplate reader at 562 nm. The optical density of blank dimethyl sulfoxide-NaOH was subtracted from all wells. The formazan content of each well was computed as a percentage of the mean of the Teflon controls, which was taken to represent 100% biocompatibility.

Because the normality and equal variance assumptions of the raw data were violated, they were logarithmically transformed before analysis with 2-factor repeated-measures analysis of variance (ANOVA). Data from the IRM-positive control were excluded from the analyses. For the evaluation of the set cements, statistical analysis was performed to examine the effect of the “material,” the “immersion period,” and the interaction of those 2 factors on cell viability. For the evaluation of the eluents derived from the set cements, statistical analysis was performed to examine the effect of the “material,” the “dilution factor,” and the interaction of those 2 factors on cell viability. For each analysis, post hoc multiple comparisons were performed using the Holm-Sidak test. Statistical significances for all analyses were set at α = 0.05.

Flow Cytometry

The viability of MDPC-23 cells exposed to the set materials after different cycles of culture medium immersion was evaluated. Cells were plated at the same density as previously described. For each week, the established cells were first exposed to the respective materials for 3 days as previously described. The cells were then washed twice with phosphate-buffered saline and detached from the culture wells with 0.25% trypsin. The harvested cells were centrifuged to discard the supernatant and resuspended at 1 × 10⁴ cells/mL in 1 × binding buffer included in the Apoptosis and Necrosis Quantification Kit (Bio- tum, Inc, Hayward, CA). The cells were stained with fluorescein isothiocyanate (FITC)–annexin V (AnV) (λ₁₈₆/λ₂₄₄ = 492/514 nm, green fluorescence) and ethidium homodimer-III (Etd, λ₁₈₆/λ₂₄₄ = 528/617 nm, red fluorescence) and incubated for 15 minutes in the dark. The stained MDPC-23 cells were subjected to fluorescence-activated cell sorting (FACS) using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) to determine the percentage distribution of viable (AnV/Etd negative), early apoptotic (AnV positive, Etd negative), late apoptotic (secondary necrosis; AnV/Etd positive), and...
necrotic cells (AnV negative, Etd positive). Experiments were performed in triplicates.

Data were collected from 6 groups: cells exposed to the negative and positive controls in the first and second weeks and exposed to Quick-Set or WMTA in the first and second weeks. Because the data were normally distributed (Shapiro-Wilk test, \( P = .922 \)), homoscedastic (modified Levene test, \( P = .559 \)), 1-factor ANOVA, and Holm-Sidak multiple comparison procedures were used to analyze the data, with statistical significances set at \( \alpha = 0.05 \).

**Confocal Laser Scanning Microscopy Imaging**

MDPC-23 cells plated at 400 cells/cm\(^2\) onto cover slips (Carl Zeiss Microscopy, Thornwood, NY) in 6-well plates were established for 3 days. Transwells with the 4 material disks were then inserted into culture wells containing the plated cells. After incubating for 3 days with the materials, the cells were triple stained with Hoeschst 33342 (\( \lambda_{	ext{abs}}/\lambda_{	ext{em}} = 350/461 \text{ nm}, \text{ blue fluorescence} \)), Etd (red fluorescence), and FITC-AnV (green fluorescence). The cover slips were then mounted on slides and sealed with nail polish for imaging of the morphology of cell death (apoptosis vs necrosis) after exposure to the materials. A 2-photon confocal scanning microscope (LSM 510 META, Carl Zeiss Microscopy) coupled to a MIRA 900 Ti:Sapphire laser (Coherent Inc, Santa Clara, CA) was used for imaging. Using these fluorophores in combination, Hoechst 33342, a cell membrane–permeable, minor groove-binding DNA stain, stains the nuclei of both apoptotic and necrotic cells. However, healthy cells are stained by Hoechst stain only, but healthy cells are not stained by FITC-AnV and Etd. Apoptotic cells are stained both green and blue. Necrotic cells are stained both red and blue. Cells stained blue, green, and red represent dead cells progressing from the apoptotic cell population.

**Results**

Quick-Set and WMTA cements that were set for 24 hours were cytotoxic to MDPC-23 cells (<5% succinic dehydrogenase activity of Teflon, data not shown). After the first week of immersion in culture medium, the succinic dehydrogenase activities for Teflon, Quick-Set, WMTA, and IRM were 100% \( \pm 6.6\% \), 91.0% \( \pm 6.8\% \), 83.0% \( \pm 14.0\% \), and 0.09% \( \pm 0.5\% \), respectively. After the second week of immersion in culture medium, the succinic dehydrogenase activities for Teflon, Quick-Set, WMTA, and IRM were 100% \( \pm 4.2\% \), 100.8% \( \pm 7.2\% \), 100.4% \( \pm 3.2\% \), and 0.9% \( \pm 3.0\% \), respectively (Fig. 1A). Two-factor repeated-measures ANOVA of Teflon, Quick-Set, and WMTA over the 2 time periods indicated that both materials (\( P = .023 \)) and immersion time (\( P < .001 \)) affected the viability/proliferation of MDPC-23 cells. The interaction of these 2 factors was also statistically significant (\( P = .009 \)). Quick-Set and WMTA were more cytotoxic than Teflon but were not different from each other after the first week of immersion (\( P < .05 \)). The 2 cements were as biocompatible as Teflon after the second week of immersion (\( P > .05 \)). Both cements exhibited differences in their cytotoxicity to MDPC-23 cells after the first and second week of immersion (\( P < .05 \)).

Eluents derived from Quick-Set exhibited cytotoxicity levels that were 22.9% \( \pm 11.4\% \), 98.6% \( \pm 8.1\% \), and 98.9% \( \pm 13.4\% \) of the Teflon-negative control at 1:1, 1:10, and 1:100 dilution, respectively. Eluents derived from WMTA exhibited cytotoxicity levels that were 64.6% \( \pm 8.0\% \), 98.5% \( \pm 7.4\% \), and 100.3% \( \pm 16.5\% \) of the Teflon-negative control at 1:1, 1:10, and 1:100 dilution, respectively (Fig. 1B). Two-way repeated-measures ANOVA indicated that the type of eluent did not affect (\( P = .296 \)) the viability of MDPC-23 cells, whereas their dilution factor affected cell viability (\( P < .001 \)). The interaction of these 2 factors is not statistically significant (\( P = .079 \)). Eluents at 1:1 dilution were more cytotoxic than eluents at 1:10 or 1:100 dilution (\( P < .05 \)). There was no difference between eluents at 1:10 and 1:100 dilutions.

Flow cytometry was performed using aged disks for quantitative analysis. Representative flow cytometric, 2-dimensional dot plots of Quick-Set and WMTA aged for 1 week and 2 weeks are shown in Figure 24 through D. Statistical analysis of the percentage of viable cells for these 4 groups as well as the Teflon and IRM control groups (Fig. 2E) showed a highly significant difference among the 6 groups tested (\( P < .001 \)). Post hoc analysis indicated that both Quick-Set and WMTA had lower percentages of viable cells at week 1 (\( P < .05 \)). However, the percentage of viable cells after exposure to WMTA at week 1 was not significantly different from the Teflon-negative control (\( P > .05 \)). After immersion in the culture medium for 2 weeks, both the percentage of viable cells exposed to Quick-Set and WMTA were not significantly different from those exposed to Teflon (\( P > .05 \)).

The flow cytometric results performed were qualitatively confirmed by confocal laser scanning microscopic imaging. Cells exposed to the Teflon-negative control for the first (not shown) or second week (Fig. 3A) were mostly vital and exhibited predominantly...
blue-fluorescent nuclei without minimal signs of apoptosis or necrosis. Cells exposed to the IRM-positive control were mostly apoptotic and necrotic after the first (not shown) or second week (Fig. 3). Cells exposed to Quick-Set (Fig. 3C) or WMTA (Fig. 3D) were predominantly vital, with minimal apoptosis or necrosis. After immersion in culture medium for 2 weeks, Quick-Set and WMTA were as biocompatible as Teflon, with cells exhibiting minimal red or green fluorescence (not shown).

**Discussion**

Cytotoxicity tests are integral in the safety evaluations of products that come in contact with human tissues. Cell culture models have significantly streamlined this process because they have clearer definitions and greater agreement for what constitutes *in vitro* cytotoxicity including no cellular attachment, dramatic morphological changes, a reduction in overall viability, and adverse effects on cell proliferation (31). Admittedly, limitations exist for cell culture models, but they provide critical information before the implementation of more labor-intensive *in vivo* studies using small- and large-animal models.

In the present study, 2 complementary strategies were used to examine the viability of MDPC-23 cells in the presence of hydraulic endodontic cements. Both strategies have their merits and limitations. The first strategy (MTT assay, Fig. 1) evaluated cell metabolic activity based on the premise that dead cells are incapable of metabolizing tetrazolium salts via mitochondrial dehydrogenases involved in the citric acid cycle and the electron transport chain. Cellular damage invariably

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**Figure 2.** Representative 2-dimensional dot plots of the flow cytometry data derived from FITC-AnnV and Edt-stained MDPC-23 cells after their exposure to Quick-Set that had been immersed in culture medium for (A) 1 and (B) 2 weeks and WMTA that had been immersed in culture medium for (C) 1 and (D) 2 weeks. The dot plot represented the distribution of viable (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic cells (upper left), respectively. (E) A histogram comparing the percentage of vital cells (lower left quadrant) after exposure to the 2 cements (n = 3). Groups labeled by the same letter designators are not statistically significant (P > .05).
results in the loss of the ability of the cell to provide energy for cell function and growth. Unlike necrosis, apoptosis is an active mode of cell death that relies on sustained energy production from cell metabolism for its induction (32). Thus, tetrazolium salt–based assays may underestimate cellular damage and detect cell death only at the later stage of apoptosis when cellular metabolism is substantially reduced. Notwithstanding this limitation, metabolic activity assays are invaluable for comparing the potency of different materials in causing cell death. Although nonclonogenic metabolic activity assays may also be used for quantifying cell proliferation (ie, the number of cells that are actively dividing because proliferating cells are more metabolically active), the effects of the hydraulic cements on cell viability only are discussed. This is because assays on DNA synthesis during the S-phase of the cell cycle (eg, cell labeling with analogs of thymidine precursors) were not used in the present work.

The MTT assay results derived from the immersed materials and their eluents both indicate that the experimental calcium aluminate cement and the commercial calcium silicate cement exhibited similar cytotoxicity profiles that were far less cytotoxic than the IRM-positive control. These results are reflections that all substances can be poisonous depending on the dosage (33) and that toxicologic risks only exist in relation to the conditions under which the cells are exposed to a compound. Important issues to consider are that culture conditions are not homeostatic and there is no elimination of toxic substances as there would have been in vivo. Conversely, a living host possesses periapical defense mechanisms and the lymphatics to remove toxic substances (34). Zinc oxide–eugenol–based restorative materials had been placed directly on exposed vital pulps and did not cause long-term pulpal inflammatory responses in the absence of bacterial leakage (35). Thus, the results should be interpreted as risk estimates of the relative rate in which healing or tissue repair is likely to occur in the presence of the materials. Likewise, results of the dilution assay (Fig. 1B) imply that odontoblast-like or stem cells recruited to the vicinity of the hydraulic cements will exhibit normal metabolic activity because the concentration of the toxic components released from the hydraulic cements is diluted by circulating tissue fluids.

Unlike the first strategy, the second strategy (differential staining) examined plasma membrane alterations (permeability) that were induced by cellular damage. Other assays such as those that measure the release of cytoplasmic enzymes (eg, lactate dehydrogenase) may also be classified in this assay category although they were not used in this study. Because dyes stain individual cells, each sample has to be analyzed by flow cytometry or microscopy. In normal cells, the distribution of phospholipids is asymmetric. Plasma membrane alterations at the cell surface that occur during the initial stages of apoptosis result in the translocation of intramembranous

Figure 3. Representative CLSM imaging of cover slip attached MPDC-23 cells that were triple stained with Hoechst 33342 (blue-fluorescent vital DNA dye) (lower left image), Edt (red-fluorescent nonvital DNA dye) and FITC-AnV (green-fluorescent phosphatidylserine-binding cytoplasmic dye) after their exposure to (A) Teflon, (B) IRM, (C) Quick-Set, and (D) WMTA. Secondary necrotic cells progressing from apoptosis are indicated by open arrowheads in the red channel (upper left image) and arrows in the green channel (upper right image). The overall result after merging of the 3 channels is shown in the lower right image. A–D: Scale bar = 10 μm.
phosphatidylserine to the outer leaflet of the plasma membrane. An-V, a calcium-dependent phospholipid-binding protein, has a high affinity for phosphatidylserine. Although it does not bind to normal living cells, An-V binds to the phosphatidylserine exposed on the surface of apoptotic cells. However, necrotic cells are also labeled by An-V upon rupture of their plasma membrane. Thus, for flow cytometry and fluorescence microscopic valuation, it is necessary to control the membrane integrity of the phosphatidylserine-positive cells by double staining with membrane-impermeable DNA dyes such as Edt (Fig. 2), propidium iodide, or 7-aminomycinycin D. Cells with altered plasma membrane permeability are stained, whereas undamaged (viable) cells are not stained with dyes that do not penetrate the plasma membrane (exclusion dyes). Conversely, the bisbenzimida-zole dye, Hoechst 33342, penetrates the plasma membrane and stains DNA in both viable and nonviable cells. Thus, it is used in confocal laser scanning microscopy imaging as an indicator of the total number of cells in a field of view (Fig. 3).

It is salient to point out that cytotoxicity evaluation is a measure of the degree to which an agent possesses a destructive action on certain cells. As such, it does not stipulate a specific cell death mechanism (ie, whether cells are rendered nonviable by necrosis [“accidental” cell death] or apoptosis [programmed cell death]). Thus, additional assays for the identification of the early (eg, caspase activation) and late stages of apoptosis (eg, DNA fragmentation) were not performed for the confirmation of the exact cell death mechanism. Likewise, only the percentages of viable cells in FACS were presented and statistically analyzed (Fig. 2E). A limitation of membrane permeability assays is that the initial sites of damage by cytotoxic agents may be intracellular. These cells may be irreversibly damaged and committed to die, but their plasma membranes are still intact. Therefore, these assays have the tendency to underestimate cellular damage when compared with other methods. However, when the results of Quick-Set and WMTA were compared under the same conditions, both hydraulic cements exhibited a similar decline in the percentages of viable cells in week 1 compared with the Teflon-negative control. Moreover, the percentages of viable cells exposed to both cements were not significantly different from those exposed to Teflon in week 2. Because similar trends were seen in 2 separate strategies that examined different aspects of cell viability, one may more confidently attest that there is not enough evidence to reject the null hypothesis that there is no difference between the experimental calcium aluminosilicate cement and commercially available calcium silicate endodontic cement in affecting the viability of odontoblast-like cells.

Within the limits of the present study, it may be concluded that both hydraulic cements are capable of affecting the viability of undifferentiated murine odontoblast-like cells but possess negligible toxicologic risks after the elution of components that compromise cell metabolism or membrane permeability. Because these risks are relatively lower than those imposed by a zinc oxide–eugenol–based cement, a favorable in vitro tissue response is likely to occur more rapidly. This functional hypothesis has to be validated by correlating the present in vitro results with future in vivo data generated from small- and large-animal models. It would be of interest to further examine if there are differences between the 2 hydraulic cements in affecting MDPC-23 cells after their differentiation into full odontoblast-like cells. This may be accomplished by examining the effects of the hydraulic cements in up-regulating osteogenic gene markers in differentiated odontoblast-like cells, examining the production of alkaline phosphatase, and quantifying the amount of calcium-containing mineral depositions within the extracellular matrices using appropriate cell culture assays. Investigations of these responses are in order.

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