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Modulation of polymorphonuclear leukocyte adherence by pulpotomy medicaments: effects of formocresol, glutaraldehyde, eugenol, and calcium hydroxide

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Abstract

Activated polymorphonuclear leukocytes (PMNs) release lysosomal enzymes and toxic oxygen-free radicals into their immediate environment. The persistent activation of PMNs by pulpotomy medicaments may contribute to the chronic inflammatory changes and root resorption seen in histologic sections. The authors examined the effects of pulpotomy medicaments commonly used in pediatric dentistry on PMN adherence, the earliest observable change in PMN behavior following activation, and perhaps 1 of the most crucial. The results showed that formocresol, eugenol, and calcium hydroxide caused lysis of PMNs at high concentrations, but activation of PMN adherence at low concentrations. By contrast, glutaraldehyde did not produce PMN lysis at high concentrations, nor did it cause activation of PMN adherence at low concentrations. These findings correspond to previous histologic studies which found that formocresol, eugenol, and calcium hydroxide, but not glutaraldehyde, can cause inflammatory destruction of pulpal tissues.

The pulpotomy technique is now an accepted procedure for treating vital primary teeth with carious pulp exposures. Medicaments commonly used following a pulpotomy procedure include formocresol, zinc oxide-eugenol, and calcium hydroxide.[1] More recently, glutaraldehyde has been suggested as a better alternative to formocresol due to its lower tissue toxicity.[2] Pulpotomy medicaments are used to kill bacteria remaining in the pulp and to preserve vital root pulp. Although clinical studies on pulpotomy have reported high success rates,[3-6] histologic studies have given disappointing results,[7-8] notably chronic pulpal inflammation, necrosis, and internal resorption. Most authors have attributed the poor histologic sequelae to the lack of local tissue compatibility with commonly used pulpotomy medications.[9] In addition, systemic effects have caused concern among clinicians.[10-12]

Polymorphonuclear leukocytes (PMNs) are phagocytic cells with important roles in host defense,[13] but in appropriate and uncontrolled stimulation of these cells can lead to their accumulation in excessive numbers resulting in tissue damage.[14] Whether or not pulpotomy medicaments have the capacity to activate PMNs has not been studied. The authors postulate that stimulation of PMNs by pulpotomy medicaments may contribute to the chronic inflammatory changes seen with their use. In this study the effects of some of these medicaments on PMN adherence, the earliest observable change in PMN behavior following activation, are examined. Adherence of PMNs to vascular endothelium is a prerequisite for subsequent diapedesis and chemotaxis into the perivascular compartment, and hence of paramount importance in the initiation of the inflammatory response.[15,16]

Methods and Materials

Pulpotomy Medicaments

Pulpotomy medicaments selected for this study included formocresol, glutaraldehyde, eugenol, and calcium hydroxide, all commonly used in pediatric dentistry.

Formocresol[a] in the form of Buckley's formula (19% formaldehyde, 35% cresol) was dissolved in absolute ethanol at a concentration of 1:5 (vol/vol), and further dilutions made in medium 199. Appropriate control

[a] Creighton Pharmaceuticals: Sydney, Australia.

Table 1. Comparative Effects of Pulpotomy Medicaments on PMN Adherence

Pulpotomy Medication Dilution	*Formocresol (Buckley's)*	*PMN Adherence (% of Control ± SD)*		*Calcium Hydroxide (Calyxl)*
		Glutaraldehyde	*Eugenol*	
1:10	lysis	0‡	lysis	lysis
1:100	lysis	45.3 ± 3.4†	lysis	lysis
1:1000	lysis	108.6 ± 2.4	lysis	122.7 ± 3.0†
1:10,000	41.5 ± 7.5**	104.8 ± 0.4	88.2 ± 5.9	116.7 ± 2.9
1:100,000	114.4 ± 1.4*	ND	121.3 ± 3.8*	107.6 ± 9.5
1:1,000,000	118.1 ± 3.3*	ND	121.2 ± 5.1	103.2 ± 7.8

Incubation time with PMNs was 15 min for all medicaments while contact time with the nylon fibers was 5 min. ND = not done. ** $p < 0.01$; * $p < 0.05$; † $p < 0.02$; ‡ $p < 0.001$.

solutions also were prepared with ethanol in medium 199.

Glutaraldehyde[b] was prepared in the recommended endodontic concentration of 2% aqueous solution, and subsequent dilutions were made in medium 199.

Eugenol BP[c] was dissolved in absolute ethanol at a concentration of 1:5 (vol/vol). Subsequent dilutions were made using medium 199. Appropriate control solutions also were prepared with ethanol in medium 199.

Calcium hydroxide[d] was prepared in a stock suspension of 10 mg/ml made up in medium 199, and further dilutions made in the same medium. A fine suspension was obtained by vigorous shaking.

PMN Adherence

PMNs were purified from heparinised blood of healthy donors by a 1-step centrifugation procedure on a resolving medium[e] as previously described.[17] The PMNs were harvested from the second band at the interface, washed twice, and resuspended in medium 199. They were of > 97% purity.

The PMN adherence assay was performed using nylon fiber microcolumns as previously described.[18] Briefly, the nylon fiber microcolumns were prepared by carefully weighing out 10 mg lots of teased nylon fiber. These were placed in 100 µl disposable pipette tips so as to occupy the center 2 cm portion of the 5 cm pipette tip. PMN suspensions with or without pulpotomy agents were adjusted to concentrations between 4-6 × 10^6 cells/ml and 100 µl was delivered into each nylon microcolumn. After incubation for 5 min at 37°C and high humidity in order to allow for contact between PMNs and nylon fiber, the microcolumns were placed in a specially designed apparatus.[18] The fluid was extracted by a vacuum suction pressure of ~ 250 millibars applied for 1-2 min into disposable test tubes. The concentration of PMNs was determined in a hemocytometer and the results calculated as follows:

$$\% \text{ Adherence} = 100 - \left| \frac{\text{PMN conc. in effluent}}{\text{PMN conc. in original suspension}} \times 100 \right|$$

Results were expressed as mean ± SD of triplicate samples. In some experiments, the results were expressed further as a percentage of control and calculated as follows:

$$\% \text{ of control} = \frac{\% \text{ adherence of test sample}}{\% \text{ adherence of control sample}} \times 100$$

The Student's *t*-test was used for statistical analysis of the results.

Viability Studies

The viability of PMNs was determined by the trypan blue dye exclusion test.[19] Briefly, the PMN suspensions were incubated with 2% trypan blue for 5 min, and the percentage of stained cells assessed by microscopy.

Results

Effects of Varying Concentrations of Medicaments on PMN Adherence

The results show that with the exception of glutaraldehyde, incubation with high concentrations of pulpotomy medicaments caused lysis of PMNs. With lower concentrations, adherence of PMNs was affected markedly (Table 1).

Formocresol at an intermediate concentration of 1:10,000 caused PMN adherence to be decreased to 41.5 ± 7.5% of controls ($p < 0.01$). In contrast, at a much lower concentration of 1:100,000 it was raised to 114.4 ± 1.4% of control ($p < 0.05$). This increase in adherence was observed even at the extremely high

[b] Sigma Chemical Company: St Louis.
[c] David Craig Chemicals: Brisbane, Australia.
[d] Calyxl — Otto and Co: Frankfurt, Germany.
[e] Mono-Poly — Flow Laboratories: Virginia.

dilution of 1:1,000,000 where the adherence percentage was 118.1 ± 3.3 of control ($p < 0.05$).

With glutaraldehyde, no lysis of cells was apparent even at a high concentration of 1:10. However, PMN adherence was depressed markedly to 0% compared to control values. At the next dilution of 1:100, PMN adherence still was depressed at 45.3 ± 3.4% of control ($p < 0.02$). In contrast, at an intermediate concentration of 1:1000, PMN adherence was increased slightly (108.6 ± 2.4), but this increase was not statistically significant ($p > 0.1$). However, at a low concentration of 1:10,000 there was no significant change in PMN adherence compared to controls.

Eugenol at high concentrations of 1:10, 1:100, and 1:1000 caused lysis of PMNs. At the very low concentrations of 1:100,000 and 1:1,000,000, there was a stimulation of PMN adherence. Percentage of PMN adherence was 121.3 ± 3.8 of control ($p < 0.05$) at 1:100,000 dilution and 121.2 ± 5.1 of control at 1:1,000,000 dilution.

Calcium hydroxide at high concentrations of 1:10 and 1:100 produced lysis of PMNs. At an intermediate concentration of 1:1000, stimulation of PMN adherence was observed at 122.7 ± 3.0% of control ($p < 0.02$). At the lower concentration of 1:100,000, no significant effect compared to control was noted.

Effects of Prolonged Incubation with Low Concentrations of Medicaments

The previous sets of experiments indicated that low concentrations of formocresol, eugenol, and calcium hydroxide caused stimulation of PMN adherence. Initial stimulation followed by depressions is a well-known response of PMNs following activation by various stimuli.[20] To determine if this activation-deactivation phenomenon is evident upon stimulation with pulpotomy medicaments, concentrations of medicaments producing stimulatory effects on PMN adherence were selected and incubated with PMNs for varying time periods. Figure 1 shows that the activation-deactivation phenomenon was observed clearly with formocresol, eugenol, and calcium hydroxide.

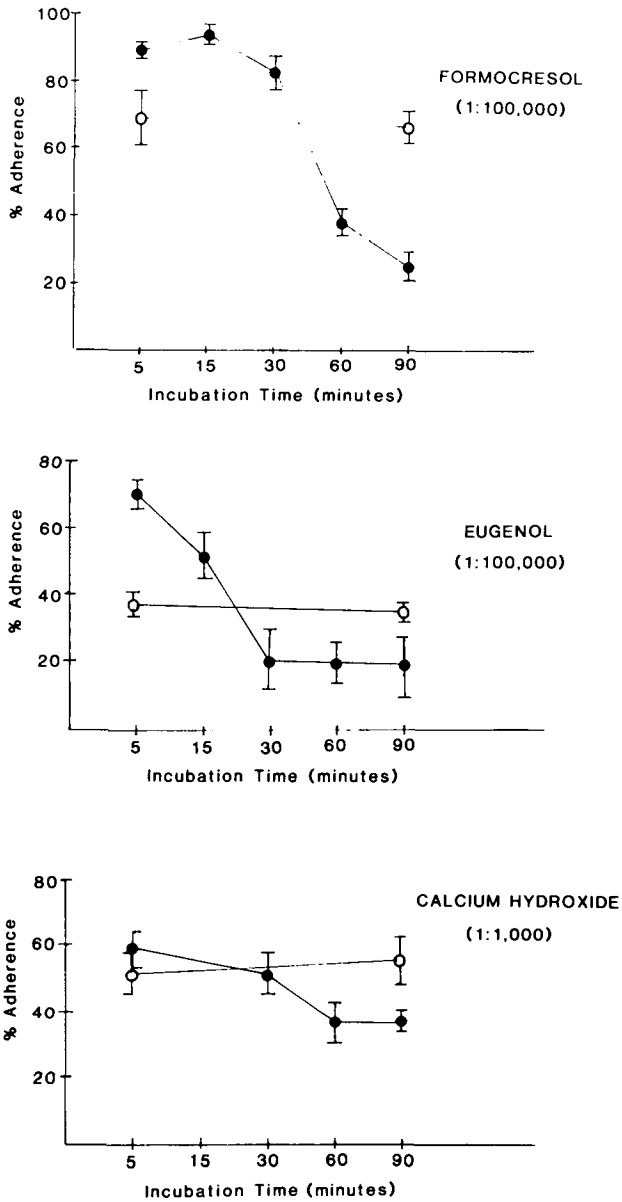

FIG 1. Effect of prolonged incubation time with high dilutions of pulpotomy medicaments on PMN adherence. The effect of each medicament was studied with PMNs obtained from a different donor. Experiments were performed in triplicate (mean ± SD) and results shown as closed circles (medicament treated) and open circles (untreated PMNs).

Effects of Formocresol and its Constituents on PMN Adherence

Since formocresol is composed of 19% formaldehyde and 35% cresol, it is pertinent to determine the individual effects of each of these components. Stock solutions of 19% formaldehyde and 35% cresol were made by using medium 199 and absolute ethanol, respectively. These solutions were diluted further in medium 199 to obtain concentrations of 1:10,000. Solutions of formaldehyde, cresol, and formocresol, all at a concentration of 1:10,000 and appropriate controls, with and without ethanol, were incubated with PMNs at 37°C for 15 min (Fig 2). Formocresol and formaldehyde at similar dilutions resulted in a comparable decline in PMN adherence. In contrast, cresol alone did not alter PMN adherence. It was necessary to use alcohol as a solvent in these experiments, but alcohol at this low concentration did not alter PMN adherence. Thus, it is the formaldehyde component of the formocresol that is responsible for the effect on PMN adherence.

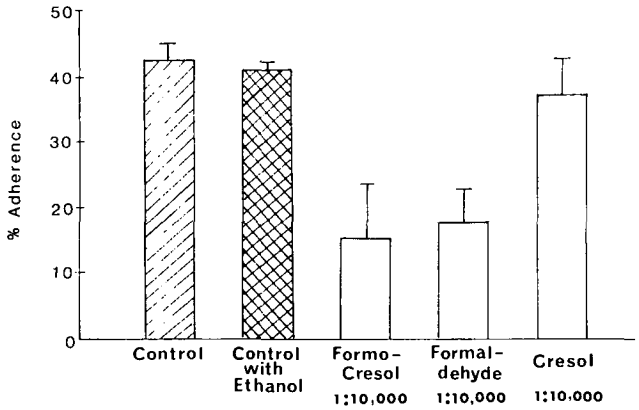

FIG 2. Effects of formocresol and its constituents on PMN adherence. Experiments were performed in triplicate and results shown as mean ± SD.

Effect of Medicaments on Phorbol Myristate Acetate (PMA)-Stimulated PMNs

Phorbol myristate acetate[f] is a derivative of croton oil with stimulatory effects on immune cells, including PMNs.[21] To determine if PMNs treated with inhibitory concentrations of pulpotomy medicaments could respond to PMA stimulation, formocresol at 1:10,000, eugenol at 1:1000, and glutaraldehyde at 1:100 dilutions were used in the next set of experiments. PMNs first were incubated with PMA (0.01 μg/ml) for 5 min, and then for another 15 min after the addition of medicament. Appropriate controls without PMA also were included in this set of experiments. The results are shown in Figure 3. PMA increased the percentage of PMN adherence. In contrast, no significant increase in PMN adherence was observed in the presence of formocresol at a concentration of 1:10,000. Similar trends were observed in separate sets of experiments using eugenol at 1:1000 and glutaraldehyde at 1:100. In these experiments, PMA stimulated the percentage of PMN adherence significantly in the controls, but failed to do so in the presence of the pulpotomy medicaments.

Viability Studies

To exclude the possibility that alteration of PMN adherence is due to loss of cellular viability, the trypan blue dye exclusion studies were performed on PMN incubated with medicaments at high and low concentrations. For each medicament concentration, viability counts were determined after incubation periods of 15 and 90 min. The results indicate that > 97% of PMNs were viable in all cases after prolonged incubation with the medicaments (data not presented).

[f] Sigma: St Louis.

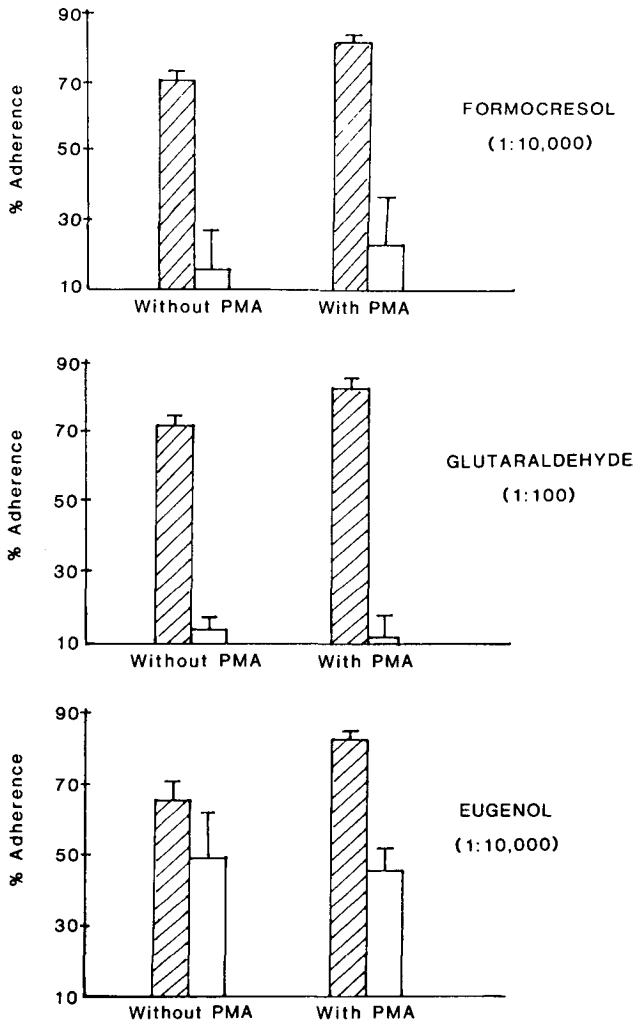

FIG 3. Effects of pulpotomy medicaments on PMA-stimulated PMNs. The effect of each medicament was studied with PMNs obtained from a different donor. Experiments were performed in triplicate (mean ± SD) and results shown as open columns (medicament treated) and closed columns (untreated PMNs).

Discussion

The results of the present studies on the effects of pulpotomy medicaments on PMN adherence demonstrate a clear correlation with recognized histologic changes seen with the use of these medicaments. With formocresol as the pulptomy medicament, a zone of fixation usually is evident where the pulp is in direct contact with the medicament. Farther away, where the concentration of formocresol is decreased, there is a zone of poor cellular definition and necrosis. Apical to this is a zone of chronic inflammation which

blends into normal tissue.[22] Histologic sections of teeth treated with calcium hydroxide or eugenol also show a zone of tissue necrosis adjacent to these medicaments followed by a zone of chronic inflammation apically.[23,24] In contrast, glutaraldehyde produces a zone of tissue fixation where it is in direct contact with the pulp, while apical to this is a zone of normal tissue with few inflammatory cells,[25,26]

In the present studies, lysis of PMNs was observed with high concentrations of formocresol, eugenol, and calcium hydroxide, but not glutaraldehyde. Of greater interest is the finding that low concentrations of formocresol, eugenol, and calcium hydroxide, *but not glutaraldehyde,* produced significant stimulation of PMN adherence. This finding corresponds well to the histologic observation of inflammatory changes in the apical zones of the pulp after the use of these 3 medicaments, where the concentrations of the medicaments are low. Stimulation of PMNs results in increased adherence, followed by diapedesis and migration of these cells to the inflammation site, where they release toxic oxygen-free radicals and lysosomal enzymes.[14] The resultant tissue damage, and the persistence of these medicaments around the pulp, would lead to the development of chronic inflammation around the pulp and subsequent tooth loss. In this regard, others have shown that pulp tissue altered by formocresol evoked a specific immune response, both humoral[27] and cell-mediated,[28] and this also may contribute to the chronic inflammatory changes following the use of this medicament.

Conclusions

Currently available pulpotomy medicaments are far from ideal. Three of the 4 studied (formocresol, eugenol, and calcium hydroxide) produce tissue necrosis and lysis of PMNs at high concentrations. At low concentrations (as low as 1:100,000 or 1:1,000,000 dilutions), the same 3 medicaments stimulate PMN adherence and may contribute to the chronic inflammatory changes seen with their use. Only glutaraldehyde appears to produce tissue fixation without causing tissue necrosis at high concentrations. Although it depresses PMN adherence at intermediate concentrations, it does not seem to stimulate PMN adherence and cause inflammatory tissue damage at low concentrations.

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