The purpose of this study was to investigate the effect of calcium hydroxide on substrate adherence capacity of rat inflammatory macrophages to determine if calcium hydroxide can alter macrophage function. Inflammatory macrophages were obtained from Wistar rats and resuspended in RPMI-1640 medium. Substrate adherence capacity assays were carried out in Eppendorf tubes for 15 min of incubation at 37°C in a humidified atmosphere of 5% CO₂. The adherence index (AI) was calculated. Results showed that calcium hydroxide decreased substrate adherence capacity of inflammatory macrophages in a time and dose-dependent manner. The lowest calcium hydroxide concentration that caused a significant inhibition of AI was 1 mM (p < 0.05), and the concentration of calcium hydroxide that caused half-maximal inhibition (IC₅₀) was 1.54 mM (p < 0.01). We conclude that calcium hydroxide decreased substrate adherence capacity of macrophages. When adhesion as the first step in the phagocytic process and in antigen presentation is taken into account, calcium hydroxide could inhibit macrophage function and reduce inflammatory reactions in periapical tissues or in dental pulp when it is used in root canal therapy or in direct pulp capping and pulpotomy, respectively. Moreover, this effect could explain, at least in part, the mineralized tissue-inducing property of calcium hydroxide.

A number of investigators have advocated the use of calcium hydroxide as a root canal filling material (1, 2). Moreover, the American Association of Endodontists Ad Hoc Committee on Treatment of the Avulsed Tooth recommended that endodontic therapy of an avulsed, replanted, mature tooth must include long-term calcium hydroxide treatment (3). According to these and other studies, calcium hydroxide when placed in contact with the pulp wound induces mineralized tissue and promotes apical closure (4), favorably influences the local environmental at the bone resorption site promoting healing of the periapical tissues (5, 6), and is antibacterial (7). However, the mechanisms by which calcium hydroxide produces some of its effects are not well known.

On the other hand, it has been demonstrated that inflammed periapical tissues contain a variety of immunocompetent cells, with macrophages predominating (8, 9). Macrophages are implicated in bone resorption (10) and play an essential role in the pathogenesis of human periapical pathosis (8, 11).

Macrophages, elicited nonimmunologically with caseinate or glycogen by washing the peritoneal cavity of rats with 0.9% NaCl 4 to 5 days after intraperitoneal injection of 5 ml of 6% sodium caseinate, are described as "casein elicited macrophages" or "inflammatory macrophages" (12, 13). Such cells have many chemical and biochemical characteristics similar to those specifically activated by immunologic mechanisms in vivo (12). Although molecular mechanisms involved in macrophage function are poorly defined (14), it is well known that adherence is the first step in the phagocytic process of inflammatory macrophages (15).

In this study the effect of calcium hydroxide on substrate adherence capacity of inflammatory macrophages was investigated to determine if calcium hydroxide could produce some of its effects acting directly on inflammatory cells, such as macrophage.

**MATERIALS AND METHODS**

**Chemicals**

Calcium hydroxide, calcium chloride, and RPMI-1640 medium were obtained from Sigma (St. Louis, MO). All other chemicals were reagent grade.

**Animals**

Male Wistar rats, aged 6 to 12 weeks, were maintained on a 12-hr light/dark cycle and allowed free access to food and water. Utmost precautions were taken so that the animals remained free from infection by environmental pathogens.

**Collection of Inflammatory Macrophages**

Peritoneal macrophages were elicited from Wistar rats by the method described previously (16). Briefly, each rat was injected intraperitoneally with 5 ml of sterile 6% sodium caseinate. Ani-
mals were killed after 4 days by decapitation and the peritoneal cavity was washed with 10 ml of cold 0.9% NaCl. After a 2-min massage, the cell exudate was removed with a syringe and centrifuged for 10 min at 250 × g at 4°C. The contaminating red blood cells were lysed with cold 0.2% NaCl. The remaining cells were then washed with 0.9% NaCl by centrifugation, resuspended in RPMI-1640 medium, counted, adjusted in the same medium at 2 to 4 × 10⁶ macrophages/ml and, immediately, used for experiments. Mean cells per rat were about 20 to 30 × 10⁶, of which 85% to 95% were macrophages by morphological criteria in Giemsa and Papanicolaou staining techniques. Viability, as determined by trypan-blue exclusion, was always greater than 95%.

**Assay of Substrate Adherence Capacity**

The quantification of substrate adherence capacity was carried out according to the technique described previously by De la Fuente et al. (17) with minor modifications. Aliquots of 180 µl of cell suspension were dispensed in Eppendorf tubes, which resemble the adherence to tissues procedure as reported by Noga et al. (18) and De la Fuente et al. (17). Calcium hydroxide (20 µl) was added to a final concentration ranging from 1 mM to 10 mM. Medium (20 µl) was added instead of calcium hydroxide to control samples. Adherence assays were performed at 5, 15, or 30 min of incubation at 37°C in a humidified atmosphere of 5% CO₂. After gentle removal (5 s in the vortex in position 5) of nonadherent cells, aliquots of 10 µl from each sample were taken, and the number of nonadherent macrophages/ml was counted in Neubauer chambers. No agglutination of macrophages was observed. When two substances were tested simultaneously, 10 µl each were added together with 180 µl of cell suspension to the Eppendorf tubes, to a final volume of 200 µl. The adherence index (AI) was calculated according to the following equation:

\[
\text{A.I.} = 100 - \frac{\text{Non-adherent macrophages/ml}}{\text{Initial macrophages/ml}} \times 100
\]

**Statistical Analysis**

All values were expressed as the mean ± SEM of the number of experiments, performed in duplicate, as indicated in the corresponding figures. The data were evaluated statistically by Student's t-test. A value of p < 0.05 (two tailed) was considered statistically significant.

**RESULTS**

Calcium hydroxide inhibited substrate adherence capacity of macrophages in all conditions tested. The inhibitory effect of calcium hydroxide was a time- and dose-dependent phenomenon. The adherence indexes obtained in control peritoneal macrophages and incubated with different calcium hydroxide concentrations (5 mM and 10 mM) for 5, 15, and 30 min are shown in Fig. 1. As can be seen, the substrate adherence capacity of control macrophages increased progressively, reaching a maximum (74.4) between 15 and 30 min. When 5 mM calcium hydroxide was added to the incubation medium, significant changes were found (p < 0.05). Higher calcium hydroxide concentration (10 mM) also decreased the AI significantly at all times tested. In subsequent experiments, incubation of cells was performed at 37°C for 15 min in a humidified atmosphere of 5% CO₂ to provide a maximal inhibitory effect of calcium hydroxide.

The concentration-effect curve for the effects of calcium hydroxide on the adherence index of inflammatory macrophages is shown in Fig. 2. The lowest calcium hydroxide concentration that caused a significant inhibition of AI was 1 mM (p < 0.05) and half maximal inhibition (IC₅₀) was obtained at 1.54 ± 0.20 mM (p < 0.01). On the contrary, calcium increased the adherence index of macrophages when it was added to the incubation medium as calcium chloride (Fig. 3).

The pH of incubation medium was determined both in the presence of calcium hydroxide and calcium chloride (Fig. 4).
The effect of higher calcium hydroxide concentrations (5-10 mM), which are similar to that used in root canals preparation, suggest pH increases progressively. On the contrary, calcium chloride did not inhibit the substrate adherence capacity of casein when calcium hydroxide was present in the incubation medium. The sensitivity of cells to a calcium hydroxide concentration as low as 1 mM, lower than concentrations of calcium hydroxide used in endodontics, and the potent inhibitory effect of calcium hydroxide could be due to the increase of pH that occurs in the incubation medium.

Macrophages play an essential role in the immune response of the host to inflammatory and infectious processes, as well as in the reparative process, but the molecular mechanisms involved are poorly defined (14). The phagocytic cell adherence to a smooth plastic surface is comparable to that taking place in animal tissues (17-19). When adhesion as the first step in the phagocytic process and in antigen presentation (15) is taken into account, calcium hydroxide could inhibit phagocytosis in macrophages and reduce inflammatory reactions in periapical tissues or in dental pulp when it is used in root canals therapy or in direct pulp capping and pulpotomy, respectively. Moreover, this effect could explain, at least in part, the mineralized tissue-inducing property of calcium hydroxide: osteoclasts and dentinoclasts, macrophage-derived cells, could decrease their functions by action of calcium hydroxide and, then, osteogenic mechanisms would predominate. We conclude that the apical extrusion of calcium hydroxide during the root canal therapy could modify macrophage functions modulating the inflammatory and reparative mechanisms involved in periapical lesions.

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FIG. 3. Concentration-effect curve for the effects of calcium chloride on the adherence index of inflammatory macrophages. Macrophages (2-4 × 10⁶/ml) were incubated at 37°C in a humidified atmosphere of 5% CO₂ in the absence (control) or presence of increasing concentrations of calcium chloride (from 1 mM to 10 mM). After 15 min the reaction was stopped and the adherence index calculated. Each point is the mean of three separate experiments performed in duplicate.

FIG. 4. Effect of calcium hydroxide and calcium chloride on the pH of incubation medium. Increasing concentrations of calcium hydroxide or calcium chloride (1-10 mM) were added to the incubation medium and, without delay, pH was determined. Each point is the mean of three separate experiments performed in duplicate.

When calcium hydroxide was present in the incubation medium the pH increases progressively. On the contrary, calcium chloride did not increase the pH.

DISCUSSION

In the present study we demonstrated that calcium hydroxide significantly inhibited the substrate adherence capacity of casein elicited macrophages. The sensitivity of cells to a calcium hydroxide concentration as low as 1 mM, lower than concentrations of calcium hydroxide used in endodontics, and the potent inhibitory effect of higher calcium hydroxide concentrations (5-10 mM), which are similar to that used in root canals preparation, suggest that the calcium hydroxide inhibition of macrophage adherence may have physiological significance in vivo at the level of periapical tissues. Moreover, calcium hydroxide concentrations found in periapical tissues attributable to its leakage during root canals treatment ranges from 1 mM to 5 mM, corresponding to the calcium hydroxide concentration that caused half maximal inhibition of AI (IC₅₀): 1.54 ± 0.20 mM.

The stimulatory effect of calcium chloride on AI suggests that the inhibitory effect of calcium hydroxide on substrate adherence capacity of macrophages does not depend on the presence of calcium ions in the incubation medium. On the contrary, the inhibitory effect of calcium hydroxide could be due to the increase of pH that occurs in the incubation medium.

Macrophages play an essential role in the immune response of the host to inflammatory and infectious processes, as well as in the reparative process, but the molecular mechanisms involved are poorly defined (14). The phagocytic cell adherence to a smooth plastic surface is comparable to that taking place in animal tissues (17-19). When adhesion as the first step in the phagocytic process and in antigen presentation (15) is taken into account, calcium hydroxide could inhibit phagocytosis in macrophages and reduce inflammatory reactions in periapical tissues or in dental pulp when it is used in root canals therapy or in direct pulp capping and pulpotomy, respectively. Moreover, this effect could explain, at least in part, the mineralized tissue-inducing property of calcium hydroxide: osteoclasts and dentinoclasts, macrophage-derived cells, could decrease their functions by action of calcium hydroxide and, then, osteogenic mechanisms would predominate. We conclude that the apical extrusion of calcium hydroxide during the root canal therapy could modify macrophage functions modulating the inflammatory and reparative mechanisms involved in periapical lesions.

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REFERENCES


A Word for the Wise

Remember we were talking once about things we often use but of whose proper name we are unaware? Sure you do! Well, consider the solidus. It is that slash that separates dates, et cetera, as in 11/22/36 for November 22, 1936. It has an interesting provenance. It was first used to mean "shilling" in English coinage where 6/2 stood for 6 shillings two pence. The symbol itself, /, was derived from the old long form of writing "s" which was ꞌ. This symbol was called a solidus because it had been used to denote the solidus, a Roman gold coin of the Emperor Constantine that began with the letter s.

Ann Newell