Effect of Histological Decalcifying Agents on Number and Stainability of Gram-positive Bacteria

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Effect of Histological Decalcifying Agents on Number and Stainability of Gram-positive Bacteria

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In tests of the effects of restorative materials on dental pulp, it is important that one evaluate bacterial contamination, and this is usually done histologically. Preceding the usual paraffin-embedding of hard-tissue specimens for microscopical investigations, decalcification is performed. To study the influence of decalcifying agents (nitric acid, formic acid, and ethylenediamine tetraacetic acid) on the number and Gram-stainability of bacteria, we used a model system consisting of suspensions of formaldehyde-fixed Streptococcus faecalis. *The Gram-positive organisms were stored in distilled water, in 4% formaldehyde solution, or in the decalcifying agents for various experimental periods. Counts were made by means of a hemocytometer, and smears were stained with the Brown and Brenn staining method. After periods which are averages for the decalcification of teeth, severe reductions of both the number and the Gram-positive stainability were found. After one week in formic acid, only one out of 15 organisms stained blue. With nitric acid and EDTA, the reductions were fewer. Since only blue-staining bacteria can be detected clearly in tissue sections, the results of these experiments indicate that, with limited numbers of organisms, the risk exists for false-negative scores for decalcified hard-tissue sections.*

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Introduction.

The current method of investigating possible irritating influences of materials on the tooth pulp is by the study of histological changes in the pulp of experimental animals after various experimental periods. Since currently-used composite filling materials are subject to polymerization shrinkage, a microspace may arise between the material and the cavity wall. If bacteria penetrate the microspace, growth can occur. Bacteria or their toxins can play a role in the initiation and maintenance of an inflammatory reaction of the dental pulp (Torstenson *et al.*, 1982; Browne *et al.*, 1983; Bauer and Henson, 1984).

The presence of bacteria in the microspace can complicate the interpretation of an observed pulpal response after cavities are filled with experimental material. Thus, it is desirable to ascertain the presence of bacterial contamination at the cavity walls and floor. This can be determined in two ways: (1) by bacteriological methods, and (2) by staining histological sections with a modified Gram technique, *e.g.*, the Brown and Brenn stain (Brown and Brenn, 1931). Disadvantages of the first method are the cumbersome sterile and anaerobic working conditions while sampling is undertaken (Cox *et al.*, 1985) and the necessity of splitting the teeth to obtain one half for culturing and one half for histological processing for the study of pulpal response (Mejàre *et al.*, 1979; Bergenholtz *et al.*, 1982).

In histological sections, generally, only Gram-positive, blue-staining bacteria are observed. This is because Gram-negative, red-staining bacteria, if present, are often difficult to distinguish from tissue components. Another disadvantage of staining is that no information is obtained on their viability and pathogenicity (Mjör, 1974). However, comparison of the results of culturing from a split half and histological examination of decalcified sections leads to good agreement concerning presence or absence of micro-organisms at the pulpal wall (Mejàre *et al.*, 1979; Browne *et al.*, 1983). On the basis of this finding, the histological staining technique is most often chosen for the detection of bacteria, because of its easier performance (Qvist and Qvist, 1980; Qvist, 1985).

After fixation, the first step of routine histological processing, prior to paraffin-embedding, is decalcification of the tissue. To save time, this is sometimes done with strong acids.

The purpose of this investigation was to determine whether number and blue-stainability of Gram-positive bacteria are influenced by treatment with HNO_3 or formic acid. In addition, the influence of EDTA was studied. Bacterial smears served as the experimental model.

Materials and methods.

Fifty mL of Trypticase soy broth (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD) was inoculated with *Streptococcus faecalis* and incubated for 24 hours at 37°C. To obtain a suspension containing a sufficient number of non-dividing bacteria per mL, we inoculated Trypticase soy with colonies from a DST stock agar [Diagnostic Sensitivity Test (DST) Agar; Oxoid Ltd., Basingstoke, Hants., England] plate to an optical density of approximately 0.3. After one and two days, the optical density had leveled off at values of from 1.20 to 1.30, indicating that division had largely stopped. A culture period of 24 hours was chosen as standard. Smears of such cultures showed high percentages (95%) of Gram-positive-staining bacteria. The bacteria were dispersed by means of a whirlimixer (Fisons, Loughborough, Leicestershire, England); aliquots were then pipetted into each of nine sterile Pyrex tubes. After centrifugation for 15 min at 1500 g and removal of broth by pipetting, sterile phosphate-buffered saline solution (PBS) was added, and the bacteria were dispersed (so-called "short wash" procedure). As a control on viability, bacteria were plated on DST agar and cultured for three days at 37°C. Readings were made after one, two, and three days: All were positive. The nine tubes were then centrifuged for 15 min at 1500 g, and, after removal of PBS by pipetting, 4% neutral formaldehyde (NF) solution was added. The formaldehyde solution was removed after renewed centrifugation, and PBS was added. After dispersion, the tubes were centrifuged, and PBS was removed. Three groups (A, B, and C) of three tubes each were made, each group consisting of two tubes as controls — to the one, 4% NF solution was added; to the other, sterile distilled water — and one experimental tube. To this tube in each group was added either 5% HNO_3 (group A), 5% formic acid (group B), or 10% sterile EDTA (group C), with pH values of 0.73, 1.80, and 7.0, respectively. The contents of all tubes were dispersed and were distributed over two tubes, resulting in two equal series of nine tubes each. From the one series, samples were taken immediately — without short wash — and counted in a hemocytometer (Bürker Türk, W. Schreck, Hofheim/TS, G.F.R., 0.0025 and 0.04 mm^2, depth 0.01 mm) (count 1). The number of *S. faecalis* per mL

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was calculated. The contents of the other series of tubes underwent a short-wash procedure, after which smears were prepared which were stained (staining 1). The experimental period of the three tubes of group A (nitric acid) was one day, that of group B (formic acid) one week, and that of group C (EDTA) four weeks. These periods are similar to the times needed for decalcification of an average tooth in the respective agent. After the experimental periods, the contents of the tubes were dispersed. Samples were drawn and counted (count 2). Then, after a short wash, smears were prepared for staining (staining 2).

To follow the procedure for staining of bacteria in tissue sections, we applied the Brown and Brenn stain (Lillie, 1965) to the smears, which were stained collectively in a staining rack. Moreover, such steps as rinses and differentiations were performed for a controlled number of seconds. The percentage of Gram-positive-staining bacteria in the smears was determined by the counting of the blue-stained bacteria in a total of 1000 bacteria.

Care was taken, in preparation of the smears, that the smearing slide (at an angle of 45 degrees) was moved without any pressure over the microscope slide. Slides were coated with filtered 1:5 diluted albumin glycerol. Fixation was performed by passing of the slide through a blue flame three times, by storing of the slide in an oven at 37°C for at least two hours, or by application of a 5% neutral formaldehyde solution for 30 min. Staining results were equal after these methods: 95% blue bacteria in fresh cultures. Counts in the hemocytometer and in smears were performed in duplicate. Moreover, a separate (verification) experiment was conducted whereby the number of bacteria per mL was determined immediately after addition of NF, HNO_3, and formic acid, and after one month, one day, and one week, respectively.

Results.

Table 1 shows the percentage of Gram-positive-staining organisms in smears (columns a) and the number of bacteria per mL (columns b). Moreover, the relative number of organisms per mL, where the number immediately after addition of 4% NF is fixed at 100 (columns c), is given.

When the numbers in columns a and c in Table 1 are multiplied per experimental group, the relative number of Gram-positive-staining bacteria per mL is obtained (Table 2). When the bacteria were stored in water for one month, the number of Gram-positive-stainable organisms per unit volume was gradually reduced by a factor of 3. Continued fixation by redisperson in NF reduced the number by a factor of 2.

Addition of strongly-acid-decalcifying agents provoked a sudden drop in the number of blue-stainable bacteria by a factor of 2 or 3, observed immediately after the addition. Moreover, this drop was followed by a further decrease, finally resulting in a reduction by a factor of 4.5, one day after addition of nitric acid, and by a factor of 15 after one week in formic acid. Addition of the neutral pH decalcifying agent EDTA caused a final reduction in the number of Gram-positive-stainable bacteria by a factor of 2.5 after one month, which is a reduction similar to that observed after one month in distilled water. However, contrary to results with distilled water, a slight drop was observed with EDTA immediately after addition. The percentages of Gram-positive-stained *S. faecalis* in smears ranged from 89% (immediately after addition of water or neutral formaldehyde) to 67% (after one week in formic acid). Therefore, the most severe reduction in stainability was by one-quarter. Those bacteria not staining Gram-positive were red or unstained, but were structurally observable bacteria. However, the reductions in the relative numbers of blue-stainable organisms per mL (Table 2) by the factors mentioned above are mainly explained by decreases in the numbers of observable bacteria, *i.e.*, the number $\times$ 10^6 per mL (Table 1, column b). Reductions by one-half, two-thirds, and nine-tenths, roughly, were seen at the end of the experiment using the respective decalcifying solutions.

Qualitatively, we noted the presence of debris after the addition of decalcifying agents, and/or with the lapse of time during the counting with the hemocytometer, or in the smears. The debris did not interfere with counting.

In a verification experiment, we again studied neutral formaldehyde, nitric acid, and formic acid. The numbers of organisms relative to the number in the reference group (again, 4% NF immediately after addition) essentially corroborated the results represented in Table 1.

Discussion.

In the present study on the influence of decalcifying agents on the number and stainability of bacteria, a model system was used. This system, using smears of a suspension of *S. faecalis*, allowed for the bacteria present to be quantified by hemocytometer-counting. The investigation was expedited by the

TABLE 1

PERCENTAGES OF GRAM-POSITIVE-STAINING *S. faecalis*, NUMBER OF ORGANISMS PER mL, AND NUMBER PER UNIT VOLUME RELATIVE TO THAT IN A CHOSEN GROUP (4% NEUTRAL FORMALDEHYDE, IMMEDIATELY AFTER ADDITION) AT VARIOUS PERIODS AFTER ADDITION OF THE LIQUID STUDIED

Liquids added to 4% NF-fixed Bacteria	Experimental Periods after Addition of Liquids: Immediately after Addition (staining 1, count 1)			1 day (staining 2, count 2)			1 week (staining 2, count 2)			1 month (staining 2, count 2)		
	a	b	c	a	b	c	a	b	c	a	b	c
4% NF	89[1]	7.7[1]	100	85	7.4	96	85	5.8	75	77	4.4	57
dist.water	89[1]	7.8[1]	101	85	7.2	94	84	5.2	68	72	3.5	45
5% HNO_3	80	3.9	51	73	2.1	27		—			—	
5% HCOOH	77	3.2	42		—		67	0.7	9		—	
10% EDTA	85	7.2	94		—			—		71	3.8	49

[a]Percentage of Gram-positive-staining bacteria in smear.
[b]Number of organisms $\times$ 10^6 per mL.
[c]Relative number of organisms per mL, where the number immediately after addition of 4% NF is fixed at 100.
—Not determined.
[1]Mean of two samples from each of three tubes; all others two samples from one tube.

TABLE 2
RELATIVE NUMBER OF GRAM-POSITIVE-STAINING BACTERIA PER mL

Liquids added to 4% NF-fixed Bacteria	Experimental Periods after Addition of Liquids			
	Immediately after Addition	1 day	1 week	1 month
4% NF	89	82	64	44
dist. water	90	80	57	32
5% HNO_3	41	20	—	—
5% HCOOH	32	—	6	—
10% EDTA	80	—	—	35

omission of histological processing, and avoided the confusion of Gram-negative (red) bacteria with tissue components.

S. faecalis was chosen, since others, such as *Streptococcus mutans* and *Lactobacillus spec.*, are difficult to count or form long chains. Although several other steps exist in the routine histological processing of tissue specimens which might influence the stainability of bacterial cells, preliminary data indicated that this was not the case.

Addition of acid-decalcifying solution, nitric acid, or formic acid, to washed and fixed bacteria appeared to exert two effects (Table 1): (1) We observed an immediate and severe drop in the numbers of structurally intact — and thus counted — bacteria, accompanied by a mild reduction of the Gram-positive-stainability; and (2) a gradual decrease was seen in the numbers of organisms with time, also accompanied by some loss of Gram-positive-stainability.

Regarding the severe reduction in number, it is conceivable that the sudden exposure to the strong acids made the cell wall swell so strongly that some organisms disintegrated. Moreover, swelling may have been facilitated by the preceding short-wash step, where much of the formaldehyde which had reacted with the proteins during fixation would have been washed out. In agreement with this explanation is the observation of much debris during hemocytometer-counting and in the smears. Other, somewhat more resistant, bacteria could have swollen less, thus retaining their integrity. But in these organisms the porosity of the cell wall could have increased to such an extent that Gram-negative-staining resulted, Gram-positive-staining being dependent, among other things, on pore size of the bacterial cell wall (Beveridge, 1981; Horobin, 1982; Beveridge, 1983). The second effect, also accompanied by some loss of Gram-positive-stainability, was observed after addition of all liquids investigated. This was interpreted as a rate-controlled hydrolysis of substances, possibly of the bacterial cell wall.

When the bacteria were stored in distilled water, the relative number per mL was reduced to 45 after one month; with storage in 4% NF, reduction was only to 57. Also, the percentage of blue-staining was higher with NF than with distilled water. Using rat liver cells, retention of proteins has been shown immediately after 4% formaldehyde fixation (Ostrowski *et al.*, 1961). When formaldehyde fixation also retains proteins in the bacterial cell wall, the spatial structure of substances containing proteins may thus be maintained, which can result in higher numbers of organisms per mL and better Gram-positive-stainability with 4% NF when compared with distilled water at any point in time. EDTA appeared to take an intermediate position between the acids and distilled water or NF.

Although nitric acid is a stronger acid than formic, which would favor swelling, its influence immediately after addition was surprisingly less severe (Table 1). This result was not reproduced in the verification experiment; however, the mutual proportions of all other data confirmed the results shown in Table 1.

The net influence of the decalcifying agents studied on number and stainability of the Gram-positive *S. faecalis* is represented in Table 2.

At the end of the decalcification time, roughly one out of three stained blue — and thus could be observed in tissue sections — by means of EDTA, with nitric acid one out of every four or five stained blue, and with formic acid, only 1 out of every 15 bacteria was observable. Under the assumption that this also applies to tissue sections containing bacteria, the number of teeth for which the diagnosis ''infected'' will be given would be reduced. This would not be too important where extensive accumulations of bacteria are present, but when few bacteria are present in a scattered occurrence, the danger of false-negative scores arises when a limited number of Brown-and-Brenn-stained sections is studied per tooth.

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