Two-hour bacterial colonization of dental luting cements in vivo

DAG ØRSTAVIK & JON ØRSTAVIK

NIOM, Scandinavian Institute of Dental Materials, Oslo and Departments of Microbiology and Prosthetic Dentistry, Dental Faculty, University of Oslo, Oslo, Norway

Ørstavik, D. & Ørstavik, J. Two-hour bacterial colonization of dental luting cements in vivo. *Acta Odontol. Scand.* 1981, 39, 115 – 123

Test specimens of zinc phosphate, polycarboxylate, zinc oxide-EBA, silico-phosphate and resin cements were carried in the mouth for 2 hours. The bacteria adhering to the cement specimens were quantified after culture on selective and non-selective, solid media. Significantly fewer bacteria could be recovered from the EBA cement than from the other cement types. All cement specimens, as well as samples from tooth surfaces, showed selective enrichment of *Actinomyces viscosus* compared with the proportion of this organism in saliva. The silico-phosphate and particularly the polycarboxylate cement were poor substrates for the adhesion of *Streptococcus mutans*. Scanning electron micrographs revealed heavy accumulations of coccoid and filamentous organisms on zinc phosphate cement surfaces. Fewer bacteria, mainly cocci, were seen on the polycarboxylate and silico-phosphate cements, whereas the micrographs of the EBA and resin cement surfaces were difficult to interpret.

Key-words: Streptococcus mutans; Actinomyces viscosus; scanning electron microscopy; humans

Dag Ørstavik, NIOM, Forskningsveien 1, Oslo 3, Norway

Secondary caries and periodontal inflammation are frequent sequelae to crown and bridge restorations (22). Both diseases are recognized to be of bacterial origin (7, 12), and bacterial specificity has been implicated for caries (7) as well as for periodontal disease (10, 20). The bacterial colonization at the junction of the tooth and the restoration may therefore be of prognostic significance for the longevity of crowns and bridges.

The space between restoration and tooth is, at least initially, filled with luting cement. The area of exposed cement may be several square millimeters (19); thus, the cement attains importance as a substrate for bacerial adhesion and colonization. Luting cements vary considerably in composition, and one might expect that differences in surface texture, surface chemistry, and dissolution of leachable components would influence bacterial adhesion and growth on the cements. Release of fluoride (11) may be of particular relevance, as fluoride may impair bacterial adhesion (18) as well as growth (9).

Previous in vitro studies have demonstrated selectivity in the attachment of oral streptococci to five different brands of luting cements (15, 16). The present study was conducted to evaluate the initial, in vivo adhesion of bacteria to the same cement brands.

Received for publication, April, 23, 1980

MATERIALS AND METHODS

Cement specimens

The five brands of luting cements used are listed in Table 1, including the abbreviations which will be used in the paper. The cements were mixed according to the manufacturers' directions in powder-to-liquid ratios approximating those given in Table 1. Freshly mixed cements were placed in Teflon® moulds and left to set under the pressure of spring clamps in a humid atmosphere for 24 h at 37°C. The EBA cement was left to set in distilled water. The moulds gave cylindrical specimens, 2 mm in height and 8 mm in diameter.

In vivo bacterial adhesion

Three series of tests were performed. In all series, 24 h old specimens were carried in the mouth for 2 h. They were placed, one on each side, in the vestibule close to the first mandibular molar. The test subjects refrained from eating, drinking, and smoking during the test period.

Series I involved one female test subject, 23 years of age. For each test 2 different cements were used, and the pairs were systematically changed to minimize intra-subject variability as a source of error. Data were obtained for a total of 30 specimens.

Series II concerned the Carb cement only: 5 subjects (one male, 35 years, and 4 females, 21–40 years) participated, and the test was carried out once with two specimens carried by each subject.

Series III was performed with the same test subject as in series I, and involved 2 specimens of all cement types.

The specimens of series I and II were evaluated for bacterial adhesion by culture techniques; the specimens in series III were examined by scanning electron microscopy. For comparison, a culture study was also performed on initial bacterial adhesion to smooth tooth surfaces with the same test subject as in series I and III. Two hours after vigorous tooth brushing without tooth-paste, the upper central incisors were sprayed with distilled water and sampled separately with cooton swabs. A sample of paraffin-stimulated, whole saliva was collected immediately afterwards. The tip of the cotton swab was aseptically cut off and transferred to 5 ml of sterile, phosphate-buffered saline (PBS: 0.14 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4). The bacteria adhering to the swab were dispersed for 15 s with a sonifier (Measuring and Scientific Equipment, London, England) operating at 20 kHz with an amplitude of 7 µm. Following serial dilutions in PBS, the bacteria were cultured and quantified as described below.

Sampling

After 2 h in the mouth, the cement specimens were removed with sterile pliers and drained free of excess saliva on a piece of sterile, absorbent paper. They were then transferred to 5 ml polystyrene tubes containing 1 ml of 0.05 % yeast extract water (YE; Difco, Detroit, Mich.), and shaken on a Whirlimixer for 5 s. Each specimen was then transferred to a second tube with 1 ml YE and shaken for 30 s. Finally, each specimen was placed in a tube containing 1 ml YE and 10 mg of glass beads (diameter 0.3 mm) and shaken for 30 s. The procedure was similar to the one described by Olsson & Krasse (14).

A sample of paraffin-stimulated, whole saliva was collected from the subjects at the same time as the cement specimens were removed from the mouth. The saliva samples were similarly diluted, cultured and quantified

Table 1. *Cements tested*

Type	Brand	Manufacturer	P/L ratio*	Abbreviation
Zinc phosphate	De Trey's improved®	De Trey Frères S.A., Zürich, Switzerland	2.2:1	ZnP
Zinc oxide – EBA	Opotow alumina EBA®	Opotow Dental Mfg. Corp., New York, USA	5:1†	EBA
Polycarboxylate	Durelon®	ESPE GmbH, Seefeld, W. Germany	1.5:1†	Carb
Silico-phosphate	FluoroThin® typhe III	SpS. White Ltd, Harrow, Middx, England	2.5:0.8	SiP
Bowen's resin	Epoxylite 9080®	Lee Pharmaceuticals, South El Monte, Calif, USA	3:1†	Resin

* Ratio of powder (g) to liquid (ml).
† Approximate values.

as was the final YE wash from the cement specimens.

Culturing conditions

In series I and II, the content of the last tube of YE was serially 10-fold diluted in PBS. Twenty-five μl aliquots from the dilution series were cultured in duplicate on 4 different solid media: horse blood agar (BA), mitis-salivarius agar (MS; Difco), sucrose-bacitracin-MS agar for *Streptococcus mutans* (MSB; 8), and the *Actinomyces* medium described by Beighton & Colman (AM; 2). The BA, MS and MSB plates were incubated for 48 h at 37°C in an atmosphere of 10 % CO_2 in air, and the AM plates for 4 d in the same atmosphere. *Strep. mutans* was identified by its colony morphology on MSB agar plates. Colonies growing on the AM medium were individually subcultured on a serum-free medium and tested for catalase activity using drops of 3 % hydrogen peroxide. Gram stained preparations of bacteria growing on AM agar consistently showed Gram-positive, branching filamentous or rod-shaped bacteria.

Plate counts were transformed to give numbers of bacteria per specimen, and altogether 5 counts were recorded for each specimen: (i) a total count, (ii) a streptococcal count, (iii) a *Strep. mutans* count, (iv) a total, *Actinomyces* count, and (v) a catalase positive (+), *Actinomyces* count.

Statistical treatment of the results

The numbers of bacteria per specimen were transformed to their $\log_{10}$ values. This made blocks of data to conform with an assumed normal distribution and permitted the use of Student's *t* test in comparative analyses. As limit for statistical significance, $\alpha = 0.05$ was chosen (3).

Scanning electron microscopy (SEM)

The specimens in series III were only brought through the first 2 washings in

YE. They were then placed in a 0.5 % solution of glutaraldehyde in the same buffer for 2 h at 4°C. After dehydration in 70, 96, and 100 % ethanol, the specimens were dried and mounted on brass stubs. They were then coated with a layer of gold and examined in a Jeol JSM 50 A scanning electron microscope (Jeol, Tokyo, Jap.).

RESULTS

Total bacterial recovery from tooth and cement

Table 2 shows the number of colony-forming units (CFU) recovered on blood agar plates. On the average, 280.000 CFU were recovered from tooth surface samples, whereas corresponding values from cement surfaces were lower, ranging from 133.000 for ZnP-cement to only 5.000 for EBA cement. However, the individual values obtained from a given material varied widely. It should be noted that, due to the difference in sampling procedure, data from tooth surfaces and cement specimens are not strictly comparable.

Statistical treatment of the data revealed that the EBA cement retained significantly less bacteria than the other cements. The differences among the other brands were not statistically significant. Furthermore, EBA cement specimens always yielded less CFU than their companions when data on bacterial recovery from paired cement specimens were compared.

Recovery of total number of streptococci and of Strep. mutans

Intraindividual findings

The number of CFU on MS agar and on MSB agar was related to the total recovery of CFU on blood agar (Table 3). All samples contained a substantial amount of streptococci. Saliva samples contained some 40 per cent; among cement samples the percentage ranged from 9 (ZnP) to 57 (EBA).

Judged by the number of colonies on MSB agar, *Strep. mutans* did not dominate among the streptococci. The ZnP cement and the Resin cement had higher percentages of *Strep. mutans* than the other cements (Table 3). The carboxylate cement appeared to serve as a particularly poor substrate for adhesion of *Strep. mutans*, since the number of colonies from this source growing on MSB agar constituted only 0.03 per cent of the total CFU on non-selective medium.

The relative number of test pieces from a given material sustaining adhesion of colony-forming *Strep. mutans* was also recorded. Again it was found that the carboxylate cement was a poor substrate, since only one out of 6 specimens harboured enough *Strep. mutans* to yield colonies on MSB agar. The number of CFU from this specimen was only 50, i.e. less than 0.2 per cent of the corresponding number of colonies found on MS agar. The other cements also had one or more test pieces which did not yield *Strep. mutans* colonies, but the frequency of specimens sustaining adhesion of these bacteria, as well as the total and the relative numbers of *Strep. mutans*, were higher than for the carboxylate brand. In comparison, all samples from tooth surfaces yielded CFU on MSB agar, and the mean number of *Strep. mutans* from this source was higher than for test samples from cement pieces.

Interindividual findings

Table 4 shows the prevalence of *Strep. mutans* on carboxylate cement as com-

Table 2. *Recovery on blood agar plates of bacteria from tooth surfaces and cement specimens. Series I – one test subject*

Source	No. of samples	Mean x 10^{-5}	Range x 10^{-5}	Mean $\log_{10}$	Pooled estimate of variance
Tooth	8	2.80	0.16–7.0	5.28	
ZnP	7	1.33	0.02–7.5	4.59	
Carb	6	0.47	0.20–1.4	4.62	0.75
EBA	6	0.05	0.02–0.19	3.55	
SiP	6	0.27	0.02– .75	4.28	
Resin	5	0.19	0.02– .65	3.93	

Table 3. *Relative occurrence † of total streptococci and of Strep. mutans. Series I – one test subject*

Source	No. of samples	Total streptococci		*Strep. mutans*		*Strep. mutans* in % of total streptococci
		mean	range	mean	range	
Saliva	15	39	12.1– 49.2	0.35	0.02– 1.37	1.4
Tooth	8	N.D.*		1.02	0.02– 5.43	
ZnP	7	9	0– 24.1	2.8	0–18.6	23
Carb	6	17	3.6– 27.5	0.033	0– 0.2	0.1
EBA	6	57	0–205.6	1.9	0– 5.0	1.1
SiP	6	35	1.3–125.0	0.88	0– 5.0	1.0
Resin	5	28	10.0– 44.8	3.0	0– 9.7	8

† Recovery on MS and MSB media in per cent of recovery on blood agar.
* N.D., not done.

Table 4. *Relationship between the proportions of Strep. mutans (Sm) on cement specimens and in saliva. Series II – 5 test subjects*

	Subjects				
	I	II	III	IV	V
Sm Carb (x10^{-2})	1.25	0.75	2.0	0	0
Per cent of total	0.28	0.015	1.865	0	0
Sm saliva (x10^{-5})	32.0	32.5	2.0	3.9	9.4
Per cent of total	0.12	0.50	0.018	0.05	0.08
'K' factor*	2.33	0.03	103.61	0	0

$$*K = \frac{\text{per cent on cement surfaces}}{\text{per cent in saliva}}$$

pared with the prevalence of the same bacterial species in saliva for 5 different test persons. The K-values recorded express the percentage of *Strep. mutans* on the cement specimens divided by the percentage of *Strep. mutans* in saliva samples from the same test person.

The data confirmed that carboxylate cement was a poor substrate for adhesion of *Strep. mutans*. For 3 test subjects very low or zero K-values were obtained. However, one subject showed similar *Strep. mutans* prevalence in saliva and on carboxylate cement, and one subject had substantially higher prevalence of this species on cement pieces than in saliva.

Recovery of total and catalase-positive actinomycetes

Actinomycetes growing on AM agar also constituted a substantial part of the bacteria adhering to tooth and cement surfaces. Table 5 shows that these

Table 5. *Recovery of actinomycetes*. Series I – one test subject*

Source	No. of samples	Total actinomycetes		Catalase + strains	
		mean	range	mean	range
Saliva	15	20	3.1– 61.1	28	0–67
Tooth	8	45	12.8–142	83	41–100
ZnP	5	21	0– 75	61	33–100
Carb	6	34	4.3–109	78	25–100
EBA	5	28	0– 50	57	36–100
SiP	6	16	5.8– 32.5	59	38–77
Resin	5	16	12.3– 25.5	59	40–75

* Total growth on AM agar expressed as per cent of total recovery on blood agar. Growth of catalase-positive strains expressed as per cent of total actinomycetes.

organisms constituted nearly half of the total CFU recovered from tooth surface samples. The corresponding values from cement surfaces ranged from 16 to 35 per cent. In comparison, 20 per cent of the salivary bacteria were able to grow on AM agar. Among the organisms growing on AM agar, catalase-positive strains, probably *A. viscosus,* were particularly effectively adsorbed to the tooth surface as well as to cement surfaces. Compared with saliva samples where *A. viscosus* amounted to less than a third of the total *Actinomyces* colonies, this species constituted some 60 per cent of actinomycetes isolated from cement pieces, and more than 80 per cent of the total *Actinomyces* colonies from tooth surfaces.

Scanning electron microscopy

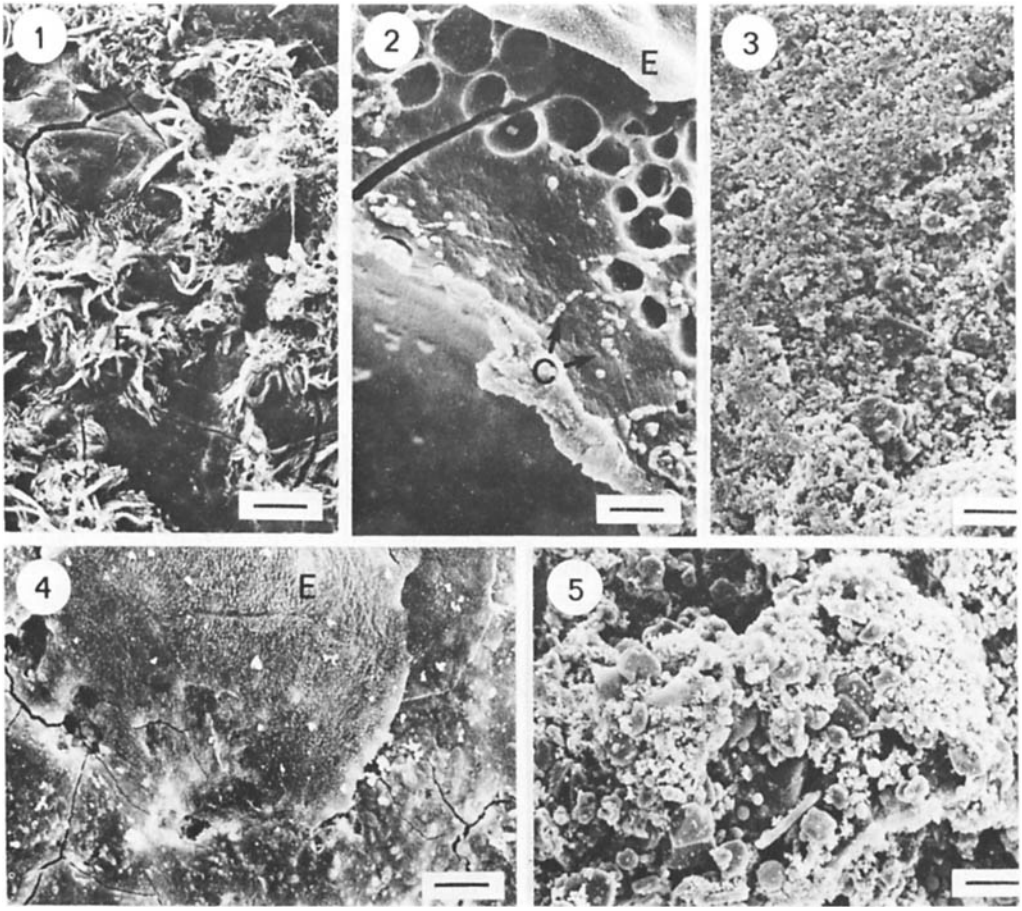
ZnP cement specimens were smooth and appeared to be almost completely covered by filamentous and coccoid bacteria (Fig. 1). Epithelial cells were present in large numbers.

A few large and many small pits, resembling air bubbles, were present throughout the surface of the Carb cement (Fig. 2). The number of bacteria appeared to be far less than on the ZnP cement, but some bacteria, predominantly cocci, clustered on the smoother parts of the surface, and a few epithelial cells could be seen.

The EBA and Resin cements had uneven, 'rocky' surfaces, and it was difficult to distinguish adhering bacteria from cement surface structures (Figs. 3 & 5). The SiP resembled the ZnP cement in surface morphology; however, very few bacteria, mainly cocci, could be seen (Fig. 4). Quite a few epithelial cells, however, were present.

DISCUSSION

The present study indicates that dental luting cements differ in their function as substrate for bacterial adhesion in vivo. The findings are in keeping with results from previous in vitro studies on bacterial adhesion to luting cements (15, 16). However, the in vitro experiments suggested more detailed differentiation among the cements concerning their adsorptive qualities, whereas in the present in vivo study, only the EBA cement adsorbed significantly fewer bacteria. This difference in results between in vitro and in vivo experiments might depend on differences in test bacteria used. Pure bacterial cultures were used in vitro, whereas the multitude of salivary bacteria contributing to adhesion in vivo may mask minor differences among the cements in their



Figs. 1 – 5. Scanning electron micrographs of cement surfaces after 2 h intraoral exposure.
Fig. 1, zinc phosphate; Fig. 2, polycarboxylate; Fig. 3, EBA; Fig. 4, silico-phosphate, Fig. 5, resin cement.
F, filamentous organisms; C, coccoid bacteria, E, epithelial cell.
Bars represent 10 μm.

ability to retain adhering bacteria. Further, the present study only recorded living bacteria, whereas the in vitro studies were carried out using techniques recording both live and dead bacteria.

An antibacterial effect of eugenol (1) and possibly of ortho-ethoxy-benzoic acid may have been the cause of the low total recovery from the EBA cement specimens. It should be noted however, that in vitro tests (15) revealed poor adhesion of a strain of *Streptococcus sanguis* to the EBA cement also with techniques counting dead as well as live cells. Therefore, the low numbers of bacteria from EBA specimens may have been caused by adhesion inhibition as well as by killing of adhering cells.

The in vitro and in vivo studies gave similar results regarding adsorption of *Strep. mutans*. The low frequency and prevalence of *Strep. mutans* on the polycarboxylate cement in vivo corresponded well with the in vitro studies, where adhesion of *Strep. mutans* to pellicle-coated polycarboxylate cement was negligible (16). In contrast, the zinc phosphate cement retained *Strep. mutans* both in vivo and in vitro. The reason for the low recovery of *Strep. mutans* from the polycarboxylate cement remains obscure. Release of fluoride from the cement may be one factor (6, 11); fluoride has been suggested to impair adhesion of *Strep. mutans* (18). Moreover, the level of *Strep. mutans* in plaque samples is lower in subjects exposed to fluoride than in subjects with little or no such exposure (13). An antibacterial effect of fluoride might also contribute to a low yield of bacteria in the present study (9). The low frequency of *Strep. mutans* on silico-phosphate cement specimens may be taken as support for such a concept, as silico-phosphate cements also release fluoride (6, 11).

The clinical implications of these general findings are not clear, as judged by the observed subject-variability in *Strep. mutans'* colonization of the polycarboxylate cement. Whereas the general trend of low adhesion prevailed with the majority of subjects tested, polycarboxylate cement specimens carried by one subject showed high prevalence of *Strep. mutans*. One may speculate that particular surface characteristics of the individual's *Strep. mutans* strains and/or the presence of salivary factors conducive to adhesion may have caused the subject differences observed.

One conspicuous feature of the present results was the relatively high numbers of catalase-positive *Actinomyces* organisms on tooth and cement surfaces as compared with the content of these bacteria in saliva. It has been suggested by others that the tooth surface may be a primary habitat of catalase-positive *A. viscosus* (4, 5); the present results seem to indicate that a high adhesion capability of this species may be an important factor in this context. Moreover, the nature of the substrate does not appear to be essential for the adhesion of the catalase-positive species, since all cement brands as well as tooth enamel behaved similarly in this respect. Also, the same trend was noted in all 5 test subjects (data not shown), suggesting that subject-variability for this phenomenon may be low.

The serial washing procedure (14) was adopted to obtain culture data on the most strongly adhering organisms, and it was the purpose of the SEM study to evaluate the cement surfaces with these bacteria. However, the results of the SEM studies were not easily related to the bacterial counts. Therefore, the effect of the final washing with glass beads represents an uncertainty in the technical procedure. Adequate SEM pictures of adherent bacteria were obtained from the zinc phosphate, polycarboxylate, and silico-phosphate cements. However, whereas the culture study did not reveal significant differences among these materials in total bacterial recovery, the SEM indicated far greater colonization of the zinc phosphate cements than of the other brands. Thus, it may be that the final washing with glass beads may have acted with different efficiency on the various cements, or that the bacteria on the zinc phosphate cement have been more firmly attached to the cement surface. The SEM pictures of zinc phosphate cement specimens showed a predominance of filamentous organisms. Moreover, the culture study indicated that this cement had lower relative numbers of streptococci than the other cements. It thus appears that zinc phosphate cement favors adhesion of a qualitatively different flora, with relatively fewer streptococci, than the other brands.

SEM micrographs of the zinc phosphate and silico-phosphate cements were strikingly different. These materials differ by the presence of silicate powder with a content of fluoride (17) in the SiP cement. The reduced colonization of the SiP cement may be related to the presence of fluoride in SiP cement through the mechanisms outlined above. Tinanoff et al. (21) have reported that fluoride treatment of enamel reduces in vivo bacterial adhesion. One may therefore speculate that fluoride in or at the surface may exert a negative influence on colonization in general as well as a selective, negative influence on colonization by *Strep. mutans*.

Acknowledgements. This study was supported in part by the Norwegian Research Council for Science and the Humanities. The authors thank Ms. Marit Kvaal for excellent technical assistance.

REFERENCES

1. Bartels, H.A. The effect of eugenol and oil of cloves on the growth of micro-organisms. Am. J. Orthodont. 1947, 33, 458 - 465
2. Beigton, D. & Colman, G. A medium for the isolation and enumeration of oral actinomycetaceae from dental plaque. J. Dent. Res. 1976, 55, 875 - 878
3. Dunn, O.J. Basic statistics: a primer for the biomedical sciences. John Wiley & Sons, Inc., New York, 1964
4. Ellen, R.P. Establishment and distribution of *Actinomyces viscosus* and *Actinomyces naeslundii* in the human oral cavity. Infect. Immun. 1976, 14, 1119 - 1124
5. Ellen, R.P., Segal, D.N. & Grove, D.A. Relative proportions of *Actinomyces viscosus* and *Actinomyces naeslundii* in dental plaque collected from single sites. J. Dent. Res. 1978, 57, 550
6. Forsten, L. Fluoride release from a fluoride-containing amalgam and two luting cements. Scand. J. Dent. Res. 1976, 84, 348 - 350
7. Gibbons, R.J. & van Houte, R.J. Dental Caries. Ann. Rev. Med. 1975, 26, 121 - 136
8. Gold, O.G., Jordan, H.V. & van Houte, J.V. A selective medium for *Streptococcus mutans*. Arch. Oral Biol. 1973, 18, 556 - 566
9. Kilian, M., Larsen, M.J., Fejerskov, O. & Thylstrup, A. Effects of fluoride on the initial colonization of teeth *in vivo*. Caries Res. 1979, 13, 319 - 329
10. Lehner, T. Cell-mediated immune responses in oral disease: A review. J. Oral Pathol. 1972, 1, 39 - 58
11. Leirskar, J. & Helgeland, K. Toxicity of some dental cements in a cell culture system. Scand. J. Dent. Res. 1977, 85, 471 - 479
12. Löe, H., Theilade, E. & Börglum Jensen, S. Experimental gingivitis in man. J. Periodontol. 1965, 36, 177 - 187
13. Loesche, W.J., Syed, S.A., Murray, R.J. & Mellberg, J.R. Effect of topical acidulated fluoride on percentage of *Streptococcus mutans* and *Streptococcus sanguis* in plaque. Caries Res. 1975, 9, 139 - 155
14. Olsson, J. & Krasse, B. A method for studying adherence of oral streptococci to solid surfaces. Scand. J. Dent. Res. 1976, 84, 20 - 28
15. Ørstavik, D. & Ørstavik, J. *In vitro* attachment of *Streptococcus sanguis* to dental crown and bridge cements. J. Oral Rehabil. 1976, 3, 139 - 144
16. Ørstavik, J. & Ørstavik, D. An *in vitro* model for studying adsorption of oral streptococci to crown and bridge cements. J. Oral Rehabil. 1980, 7, 43 - 50
17. Phillips, R.W. Skinner's Science of dental materials. W.B. Saunders Co., Philadelphia 1973
18. Rölla, G. Effects of fluoride on initiation of plaque formation. Caries Res. 1977, 11 (suppl. 1), 243 - 261
19. Silness, J. & Hegdahl, T. Area of the exposed zinc phosphate cement surfaces in fixed restorations. Scand. J. Dent. Res. 1970, 78, 163 - 177
20. Slots, J. The predominant cultivable microflora of advanced periodontitis. Scand. J. Dent. Res. 1977, 85, 114 - 121
21. Tinanoff, N., Brady, J. M. & Gross, A. The effect of NaF and SnF_2 mouthrinses on bacterial colonization of tooth enamel. TEM and SEM studies. Caries Res. 1976, 10, 415 - 426
22. Valderhaug, J. Periodontal conditions and carious lesions following the insertion of fixed prosthesis: a 10-year follow-up study. Int. Dent. J., 1980, 30, 298 - 304