Influence of a Dental Ceramic and a Calcium Aluminate Cement on Dental Biofilm Formation and Gingival Inflammatory Response

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Till Lars, Richard och Sofie

Ut audiat sapientiam auris tua, inclina cor tuum ad cognoscendum prudentiam.

Liber proverbiorum 2:2

turning your ear to wisdom
and applying your heart to understanding

Proverbs 2:2
Abstract

Dental restorative materials interact with their surrounding oral environment. Interaction factors can be release of toxic components and/or effects on biofilm formation and gingiva. In the end of the nineties, a calcium aluminate cement (CAC) was manufactured as a “bioceramic” alternative to resin composite. Dental ceramics are considered to be chemically stable and not to favour dental biofilm formation. Since the influence of aged, resin-bonded ceramic coverages is not fully investigated and the effect of CAC restorations on the dental biofilm formation and gingival response is unknown, those issues were evaluated in this thesis.

With or without oral hygiene, in clinical trials including cervical surfaces of CAC, and approximal surfaces of a leucite-reinforced bonded ceramic; biofilm growth, presence of caries-associated bacteria, clinical expressions of gingivitis, the amounts of gingival crevicular fluid (GCF) and its levels of IL-1α, IL-1β and IL-1 ra were investigated in comparison with resin composite and enamel. In addition, the unknown cytotoxic effect of specimens of CAC on fibroblasts was assessed in vitro.

With current oral hygiene a similar biofilm formation and gingival response, as evaluated, were observed at sites of CAC, resin composite and enamel. After ceased oral hygiene, more biofilm was assembled on CAC and on resin composite than on enamel. Neither with, nor without oral hygiene, biofilm formation, presence of caries-associated bacteria, clinical gingivitis and the levels of IL-1α, IL-1β and IL-1 ra differed between sites of ceramic, resin composite and enamel. Higher volumes of GCF were collected at ceramic sites compared to enamel. Fresh specimens of CAC showed the lowest cytotoxic effects on fibroblasts compared with three resin composites, zinc phosphate and glass ionomer cements.

In conclusion, the low cytotoxic effect of CAC and the limited increase in dental biofilm formation on that material compared with enamel suggest CAC to be a biocompatible dental material with respect to dental biofilm formation, presence of caries-associated microflora and gingival response. This finding, together with the observation that the influence of bonded ceramic on dental biofilm formation, caries-associated microflora and clinical gingivitis was not different from that of enamel, implicates for both CAC restorations and bonded ceramic that the need of oral hygiene and professional oral health care is not reduced.

Key words: cements, ceramics, cytotoxicity, dental biofilm, gingivitis, interleukin-1, oral hygiene, resin composite
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**Abbreviations**

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<td>CAC</td>
<td>calcium aluminate cement</td>
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<tr>
<td>CFU</td>
<td>colony forming units</td>
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<td>CSS</td>
<td>cross sectional study</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DMFS</td>
<td>decayed, missed and filled surfaces</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>GBI</td>
<td>gingival bleeding index</td>
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<td>gingival crevicular fluid</td>
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<td>GI</td>
<td>Gingival Index</td>
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<td>IL</td>
<td>interleukin</td>
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<tr>
<td>PI</td>
<td>plaque index</td>
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<td>PPD</td>
<td>probing pocket depth</td>
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<tr>
<td>PVC</td>
<td>polyvinyl chloride</td>
</tr>
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<td>QHI</td>
<td>Quigley Hein plaque index</td>
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<tr>
<td>ra</td>
<td>receptor antagonist</td>
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<tr>
<td>SBI</td>
<td>sulcus bleeding index</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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Preface

This thesis is based on the following papers, which are referred to in the text by their Roman numerals I-V:


V. Konradsson K, Claesson R, van Dijken JWV. Dental biofilm, gingivitis and interleukin-1 adjacent to approximal sites of a bonded ceramic. *Submitted*

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Introduction

Dental biofilm

Microbial biofilms develop on solid surfaces in a non-sterile aqueous solution (ten Cate 2006). The biofilm which covers the surfaces of the oral cavity, is protective by preventing colonization of exogenous microorganisms (Marsh and Bradshaw 1997, Socransky and Haffajee 2002), but it is also a basic prerequisite for developing caries (Fehr von der, and Theilade 1970) and plaque-induced gingivitis (Löe et al. 1965). The complex oral biofilm consists of bacteria, embedded in a matrix of their metabolic products and host-derived components (Marsh 2004, Marsh 2006, Socransky and Haffajee 2002). About 1000 different oral bacterial species are identified, half of which is cultivable (ten Cate 2006). The dental plaque on the non-shedding tooth surfaces is redefined as the dental biofilm (Rudney et al. 2000, Socransky and Haffajee 2002).

The formation of the dental biofilm, involves overlapping phases. Initially, the acquired pellicle, a conditioning film of glycoproteins and proteins, originated from host and bacterial molecules in the saliva and GCF adsorbs to the tooth surfaces (Lendenmann et al. 2000, Rüdiger et al. 2002, Rudney et al. 2000). Colonization of bacteria occurs by adhesion, interaction/communication and growth/metabolism. In the passive transport of oral bacteria to the tooth surface, early bacterial colonizers adhere to the pellicle by non-specific, weak, long-range interactions and specific adhesin-receptor mediated attachments. Other bacteria (co)adhere to already attached cells and multiply (Marsh 2004, Marsh 2006, Teughels et al. 2006). The various exopolysaccharides, which are synthesized by bacteria, confer to the physical and environmental characteristics of the biofilm (Sutherland 2001).

Healthy gingiva is associated with a few layers of the gram positive cocci and rods. Early colonizers are able to withstand the high oxygen concentrations and to resist natural removal mechanisms into the oral cavity (Sbordone and Bortolaia 2003). Species involved are streptococci, such as S. mitis, and S. oralis, and actinomyces, such as A. naeslundii (Li et al. 2004, ten Cate 2006). A pivotal role of actinomyces is to form a mixed biofilm with lactobacilli, which do not form biofilms on their own (Filoche et al. 2004). During biofilm formation, the local microenvironment shifts due to pH changes, the supply of nutrients and decreased oxygen levels.
(Marsh 1997, Marsh 2003) which enable microbial succession (Socransky and Haffajee 2005). Intermediate, *i.e. Fusobacterium*, and late colonizers, among them periodontitis-related bacteria, are unable to directly stick to hard tooth surfaces. They will attach when the physiological conditions in the biofilm are more likely to support their growth and survival (Sbordone and Bortolaia 2003, Socransky and Haffajee 2005, ten Cate 2006). After 2-3 weeks of undisturbed growth a mature and relatively stable microflora is established (Marsh and Bradshaw 1995).

The ecological balance of the biofilm is sustained due to bacterial interactions. The microbial homeostasis can, however, be changed by a more substantial alteration of the bacterial and host products. Metabolic waste products affect the underlying teeth or the surrounding periodontal tissues (Marsh 2003). Penetration of those tissues confers to an environmental and microbial shift, and caries and periodontal diseases can be established (ten Cate 2006).

**Gingival inflammation**

The gingiva is a specialized structure whose structure and function interact with the other periodontal components *i.e.* periodontal ligament, cementum and alveolar bone (Bartold 2006). Principally, periodontal diseases are infections caused by bacteria in the biofilm that colonize the tooth surface at or below the gingival margin (Socransky and Haffajee, 2002). Nevertheless, oral health or disease depends on the host and the microbial community as a whole (Jenkinson and Lamont 2005). The periodontal diseases are recognized as ‘eco-genetic’ diseases. The role of bacteria, host immune and inflammatory responses, gene polymorphisms, local and systemic environmental factors and various medications are able to impact the structure and function of the connective tissues. In interplay with those factors, the inflammatory process comprises a series of molecular and cellular events that lead to a host response as a defence against trauma and microbial invasion. Eventually, the host response is involved in processes of repair of tissue compartments injured by the direct effect of invaders or trauma or host’s own systems (Bartold 2006).

Broadly, gingival inflammation, *i.e.* gingivitis, can be described as the complex inflammatory host response on various stimuli to the gingiva. Gingival diseases are either plaque-induced or not primarily associated with dental plaque (Armitage 1999). Most prevalent is plaque-induced gingivitis, which is associated with an organized biofilm of several
bacterial cell layers (100-300). The species involved vary. At development of gingivitis the number of gram negative bacteria increases and anaerobic bacteria appear by microbial succession, such as *Fusobacterium nucleatum*, *Campylobacter gracilis*, *Tannerella forsythia*, *Capnocytophaga* and *Prevotella species* (Rüdiger *et al.* 2002, Sbordone 2003, Socransky and Haffajee 2005). Besides, gingivitis favours the rate of dental biofilm formation (Daly and Highfield 1996, Ramberg *et al.* 1994).

From experimental gingivitis studies of abstained oral hygiene for four weeks, it was concluded that increased quantities (Löe *et al.* 1965) and an altered quality of the biofilm (Theilade *et al.* 1966) are followed by clinical signs of gingivitis, manifested by gingival redness, swelling and bleeding. The development of gingivitis is directly associated with the rate of dental biofilm formation which varies to a great extent between different individuals. The plaque-induced gingivitis is reversible. After about one week of oral hygiene the gingivitis fades off (Löe *et al.* 1965, Theilade *et al.* 1966).

Oral bacterial infectious diseases can be treated in a physical, antimicrobial and ecological way (Socransky and Haffajee 2002). A good oral hygiene, by removing the dental biofilm, is still the most effective procedure for control of the biofilm-related diseases (Axelsson and Lindhe 1981, Jenkinson and Lamont 2005, Sbordone and Bortolaia 2003).

*The histopathology of gingivitis*

The development of a gingival inflammatory infiltrate in the marginal connective tissue was studied by Page and Schroeder (1976). Within a few days of dental biofilm accumulation, the acute inflammatory response on bacterial components and products results in an initial gingival lesion. The initial response comprises a release of proinflammatory mediators from stimulated epithelial cells, activation of endothelial cells and dilatation of the vessels. Concomitantly, the flow of the gingival crevicular fluid (GCF) enhances (Zappa 1995). Proteases, originated from epithelial cells, fibroblasts, and leukocytes, degrade the surrounding tissue to an infiltrate which comprises about 10 % of the marginal connective tissue. Neutrophilic or polymorphonuclear leukocytes are responsible for the major tissue degradation. Those have many receptors on their surfaces recognizing and fighting bacteria by antimicrobial substances and phagocytosis. The migration of neutrophiles from the vessels through the connective tissue and epithelium to the sulcus is stimulated by
chemotactic signals from bacteria and host cells, and by adhesion molecules on the endothelial cells and neutrophiles (Page 1999). The initial stage of gingivitis is a sub-clinical condition, without obvious clinical expressions. After one week, the vessel permeability increases further, resulting in clinical signs of redness and swelling. The junctional epithelium begins to proliferate. Abundant amounts of T-lymphocytes and macrophages are available in the infiltrate enclosing about 15 % of the connective tissue in which the loss of collagen increases. This early lesion can be changed over to an established lesion by further degradation of the connective tissue, expanded changes of the vessels, increased leukocyte migration, enhanced GCF flow and abundant amounts of leukocytes embedded into the gingival tissue. At this stage, the clinical expressions are apparent in redness, swelling and bleeding. Subject to a persistent biofilm, a chronic gingivitis may be established. In most individuals, the lesion is delimited to the gingiva due to the host response. However, in others, the connective tissue degradation is followed by effects on the periodontal ligament and bone, causing an irreversible lesion due to attachment loss, i.e. periodontitis (Zappa 1995).

**Gingival crevicular fluid**

The GCF is found in the sulcus or periodontal pocket between the tooth and marginal gingiva. In gingival health, the small amounts of the fluid represent the transudate of gingival tissue interstitial fluid passing through the junctional epithelium, but in course of periodontal disease the fluid is transformed into an inflammatory exsudate (Alfano 1974, Griffiths 2003). GCF is a complex mixture of substances derived from serum, leukocytes, periodontal cells and oral bacteria. The host-derived substances in the GCF include antibodies, cytokines, enzymes and tissue degradation products (Uitto 2003). Prior to the apparent clinical signs of gingivitis, the dental biofilm formation provokes a sub-clinical, gingival inflammatory response, associated with an increase in GCF flow (Löe and Holm-Pedersen 1965). Measurements of the enhancement of the GCF flow are a useful indicator of gingival inflammation (Poulsen et al. 1979). The contents of the GCF reflect bacterial activity in the gingival pocket and inflammatory events taking place in the gingival tissues (Eley and Cox 1998, Embery and Waddington 1994, Giannobile et al. 2003).
Cytokines

Cytokines are low-molecular-weight proteins which induce and maintain an inflammatory response. They can be synthesized by most of the host cells in response to bacteria and their products (Dinarello 2000). Bacterial endotoxin i.e. lipopolysaccharides, are capable to stimulate polymorphnuclear leukocytes to release proinflammatory cytokines (Zappa 1995) and also their inhibitors (Yoshimura et al. 1997). Cell stressors of non-microbial origin can also stimulate the synthesis of cytokines. These are divided into different groups due to their biological activities, which can be altered in various biological processes. One group of cytokines is named interleukins (IL). Other cytokines have retained their original description, such as tumor necrosis factor (TNF). Proinflammatory cytokines promote inflammation, whereas the gene expression of those is suppressed by anti-inflammatory cytokines. Inflammation mediated by cytokines is a result of a cascade of gene products usually not produced in healthy individuals. IL-1 and TNF act synergistically to stimulate adhesion molecules on leukocytes and endothelial cells, to initiate other inflammatory mediators, to induce expression of matrix metalloproteinases, to stimulate apoptosis of matrix producing cells and to enhance bone resorption (Graves and Cochran 2003). IL-4, IL-10 and IL-13 are some of the anti-inflammatory cytokines suppressing IL-1, IL-8, TNF and vascular adhesion molecules (Dinarello 2000).

Interleukin-1

IL-1 is one of the major inflammatory mediators and appears early in the inflammatory process. It has different effects on several cell types including stimulation or suppressing of other proteins. IL-1 stimulates small mediator molecules, such as platelet-activating factor and prostaglandins, which are potent vasodilators. Besides, IL-1 increases the synthesis of collagenases and stimulates T and B lymphocytes (Dinarello 2000). Many cell types are producers of IL-1 (Seymour and Gemmell 2001), but primarily producers are mononuclear phagocytes when stimulated by microbial or inflammatory agents. There are two distinct proteins of IL-1, coded by different genes, IL-1α and IL-1β. These are structurally related proteins of different amino acid sequences. Both types of IL-1 can bind to two distinct cell surface receptors, IL-1 R Types I and II, of which IL-1 R type II functions as a decoy molecule (Graves and Cochran 2003). Due to binding to the same receptor, there are no differences between the biological actions of IL-1α and IL-1β. However, IL-1α is a cell-
associated cytokine which can also act as a transcription factor, whereas IL-1β is secreted and therefore, more likely to play a role in systemic inflammation (Dinarello 2005). The IL-1 receptor antagonist (IL-1ra) exists as a unitary mechanism within the immune system to control IL-1 activity. This protein acts by binding with high affinity to the same receptors as IL-1α and IL-1β without inducing any of the biological effects (Weber and Iacono 1997, Dinarello and Wolff 1993). Production of IL-1 and IL-1 ra is differently regulated, even in the same cell.

Experimental gingivitis models have demonstrated that IL-1 (Kinane et al. 1992) or IL-1β in GCF increases during gingival inflammation prior to the onset of clinical signs of gingivitis (Heasmann et al. 1993, Zhang et al. 2002).

**Dental caries**

Dental caries is a localized destruction of susceptible dental hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates. Caries results from the interactions over time between the acid-producing bacteria, a substrate to metabolize and host factors (Selwitz et al. 2007). The multi-factorial caries disease is associated with lifestyle-relating factors, such as the frequency of sugar intake, dietary food, oral hygiene, use of fluoride products, socio-economic factors and related genetic factors (Fejerskov 1997).

Caries develops by microbiological shifts within the dental biofilm and is affected by salivary flow and its composition (Marsh 1994, Selwitz et al. 2007). The microbial shift results in an increase of the proportions of acidogenic and acid-tolerating bacteria, which can rapidly metabolize dietary sugars to weak organic acids, mainly lactic acid, and adapt to low pH with an optimal growth and metabolism. Well-known caries-associated bacteria are mutans streptococci, *S. mutans* and *S. sobrinus*, and lactobacilli, although other species may also be involved (Kleinberg 2002, Marsh 1994, Marsh 2006). Mutans streptococci adhere to the oral hard tissues and take a major part of the initiation of caries (Gibbons 1984, Liljemark and Blomquist 1996, Loeshe et al. 1986, Selwitz et al. 2007). They are also able to synthesize extracellular glucans from dietary sucrose specifically (Houte, van 1994, Paes Leme et al. 2006). The concomitant pH-fall in the dental biofilm might enhance the proportions of lactobacilli. They support a caries-inducing environment and attend in the proceeding
demineralization of the caries lesion (Crossner et al. 1989, Houte, van 1994, Kleinberg 2002). The acids cause a local pH fall below a critical value in which calcium and phosphate ions dissolve out of the tooth. The balance between this demineralization and the remineralization, through uptake of calcium, phosphate and fluoride, affects the caries development. The saliva acts as a buffer to protect against dissolution of the mineral content at pH-fall (Paes Leme et al. 2006).

**Dental materials**

Dental materials restore the anatomy and function of the tooth surfaces affected by caries. Aesthetics and concern about the biocompatibility of amalgam have increased the use of tooth-colored restorations. Common direct filling materials are resin composites, glass ionomer cements and intermediary materials between them. The poor mechanical properties of the non-resinous materials limit their use in loaded cavities (Mjör et al 1990).

Secondary caries is the main reason for replacement of those materials (Mjör 2005). Dental caries is even a primary failure of cast restorations. Therefore, an ‘ideal’ luting agent preventing dental biofilm formation and caries is required (Rosenstiel et al. 1998). Filled or unfilled resins and glass ionomer cement function as luting agents with a high adhesive potential, establishing or increasing the retention of the crowns or inlays to the abutments and maintain their integrity. The curing of some resin materials is initiated either chemically, through the application of light, or in both ways (Macorra and Pradies 2002).

*Resin Composites*

Resin composites are used frequently because of their aesthetics and physiological properties in all cavity classes with a tooth substance saving preparation technique. However, polymerization shrinkage of the resin composites occurs (Friedl et al. 2000, Gladys et al. 2001) which can cause marginal defects (Hansen 1982). Initially, substances can be released from the resin composites because of incomplete monomer-polymer conversion (Ferracane 1994). The substances are potential inducers of cytotoxic and/or contact allergic reactions (Nadarajah et al. 1997, Schedle et al. 1998, Wrangsjö et al. 2001).
Ceramics

Ceramics can be defined as inorganic, non-metallic materials, mostly as a result from high-temperature reactions. A wide definition of ceramics comprises also metal oxides and cement. The specific properties of ceramics are hardness, porosity, and brittleness. Early ceramics showed a low fracture resistance, which was improved in leucite crystals reinforced feldspathic porcelain. Ceramic onlays/crowns of the leucite-reinforced glass (Empress I), luted with a dentine bonding adhesive system and a resin composite material, have showed satisfactory clinical performance (Fradeani 1997, Dijken van 2001). Bonding of ceramics has been applied during the last decades for the manufacturing of veneers, inlays/onlays and full crowns. Dental ceramics are suggested to be chemically stable with a high biocompatibility (Schuster et al. 1996) and not to favour plaque accumulation (Chan and Weber 1986, Savitt et al. 1987, Jensen et al. 1990). However, Messer et al. (2003) implicated that “all dental ceramics should be tested for biologic safety because prediction of safety on the basis of composition or class of materials may not be reliable. In particular, newer dental ceramics should be valuated specifically and not be assumed to be equivalent to traditional feldspathic ceramic materials or other contemporary materials of the same type.”

Calcium aluminate cement

In the late nineties, calcium aluminate cement (CAC) was developed as a “bioceramic” alternative to amalgam and resin composite, intending to be directly used in Class I, II and V cavities. The CAC is inorganic and non-metallic. It is based on two essential constituents, alumina and calcium oxides and a small amount of zirconium, ferric and silicone oxides. Fused together at a high temperature, small particles of calcium-aluminates are formed, having a cement-forming potential. To start the hardening reaction the calcium aluminate tablets are brought into contact with the supplied liquid, which contains water and small amounts of lithium as an accelerator. An acid-base reaction is initiated. Water acts as a weak acid and calcium aluminate dissolves to form $\text{Ca}^{2+}$, $\text{Al} (\text{OH})_4^-$ and $\text{OH}^-$. The solutes precipitate to form a gel. Gradually, the amorphous gel changes into a crystalline phase $[(\text{CaO})_3(\text{Al}_2\text{O}_3)(\text{H}_2\text{O})]$ (Sunnegårdh-Grönberg 2004). Poor mechanical properties (Sunnegårdh-Grönberg 2004, Geirsson et al. 2004 a), and low clinical durability have recently been observed (Dijken van and Sunnegårdh-Grönberg, 2004).
Dental materials and biofilm formation

All biofilms consist of three ‘components’; the surface for attachment, the microbial community itself, and the “bulk fluid” which passes over the biofilm. Each of the three ‘components’ may be altered to local or subject level factors which may influence the microbial composition of the colonizing biofilm (Socransky and Haffajee 2005). Initially, the adhesion of saliva proteins and the composition of the pellicle can be affected by the adherent surface (Steinberg and Eyal 2002). The biofilm growth on the materials of the restored teeth can vary due to surface characteristics of the materials, i.e. surface-free energy and surface roughness. The surface roughness is likely to have a greater influence on biofilm retention than surface-free energy (Quirynen et al. 1990). Dental biofilm formation starts in cracks and grooves, and in irregularities in which the bacteria are protected. Defects and rough surfaces can promote the dental biofilm accumulation (Quirynen 1995). The surface area increases with the surface roughness and hence, the colonization. Surface roughness provides protection from shear forces and augments the difficulty in cleaning (Socransky and Haffajee 2002). Accumulation of the biofilm on dental cements and resin luting materials is mainly related to achieving and maintaining a highly polished surface which can be influenced by the high porosity of those materials (Macorra and Pradies 2002).

The initial antibacterial effect of ion-releasing dental materials is well known (Berg et al. 1990, Dijken van et al. 1997, Foley and Blackwell 2003, Morrier et al. 1998). Still, mutans streptococci tend to colonize restored surfaces to a greater extent than sound tooth surfaces (Lindquist and Emilson 1990), however, not demonstrated by others (Dijken van et al. 1991a). In addition to the properties of the restorative material, overhangs can affect the dental biofilm formation and periodontal health (Lang et al. 1998). Even presence of minute overhangs, difficult to detect clinically, predisposes for development of secondary caries, which indicates that dental biofilm accumulation is a predisposing factor (Mjör 2005).
Biocompatibility

A biomaterial is defined as a non-viable material used in a medical device, intended to interact with biological systems. The biocompatibility of a material is its ability to perform with an appropriate host response in a specific situation (Black 1999). Biocompatible materials must be free from any unacceptable risk (Schmalz 1997). The American Dental Association and American National Standard Institute (ADA/ANSI) have recommended biocompatibility testing of dental materials in order to obtain full clinical acceptance. Standards for testing dental materials, which are considered as medical devices, are described in ISO 10993-5 (1997), ISO 7405 (1999) and the ADA guidelines for posterior restorations (1996). Those are used to better compare data. If more appropriate, other tests can be chosen (Schmalz 1997).

Restorative materials interact with their surrounding biological environment. Interaction factors can be release of components from dental materials (Ferracane 1994) and the surface characteristics of the material (Quirynen and Bollen 1995). Biofilm formation on tooth restorations may also influence their stability and biocompatibility (Santerre et al. 2001). Adverse biological effects caused by dental restorative materials may be expressed clinically or sub-clinically, toxically and/or allergically (Schmalz and Garhammer 2002, Geurtsen 2000). Consequently, biocompatibility testing can not rely on a single test. A concept of biocompatibility testing is to use initial tests, such as cytotoxicity testing on a cellular level, secondary tests, such as implantation tests, and usage tests, which mirror the patient situation (Schmalz 1997). Studies on the biocompatibility of restorative materials have focused in vitro on inflammatory responses and toxic effects on fibroblasts and epithelial cells (Hanks et al. 1996, Santerre et al. 2001, Schedle et al. 1998, Schmalz et al. 2000, Wataha et al. 1994) as well as in vivo on longitudinal clinical follow-ups of the materials’ efficacy and durability (Mjör et al. 1990, Mjör et al 2000, Dijken van 1999, Dijken van et al. 2001, Dijken van and Sunnegårdh-Grönberg 2004, Sunnegårdh-Grönberg 2004).
Aims

The aim of this thesis was to evaluate the influence of a calcium aluminate cement and resin-bonded, leucite-reinforced ceramic on dental biofilm formation and gingival response.

More specifically, the aim was to study dental biofilm growth, clinical signs of gingivitis, the amounts of gingival crevicular fluid, its levels of IL1α, IL-1β and IL-1ra and, the presence of mutans streptococci and lactobacilli on those restoratives in vivo. In addition, the intention was also to study the cytotoxicity of CAC on fibroblasts in vitro.

The purposes of the five studies were as follows:

To evaluate CAC regarding plaque formation and gingival inflammation with or without customary oral hygiene, in comparison with resin composite and enamel (Paper I).

To compare CAC, resin composite and enamel with respect to adjacent levels of IL-1α, IL-1β and IL-1ra in GCF (Paper II).

To compare the cytotoxicity of the CAC with several currently used direct restorative materials (Paper III).

To examine the levels of mutans streptococci and lactobacilli in plaque 1) on approximal surfaces of resin-bonded ceramic restorations, and 2) on Class V restorations of CAC, and compare the levels with those on resin composite and enamel (Paper IV).

To investigate the influence of aged, resin-bonded, leucite-reinforced ceramic restorations on approximal dental biofilm formation and gingival inflammatory response associated with and without customary oral hygiene (Paper V).
Material and Methods

The evaluations of the CAC, which was denominated as a ‘novel ceramic filling material’ in Paper I, are described in Paper I-IV. The investigations of the resin-bonded, leucite-reinforced ceramic are reported in Paper IV-V. All papers, except for Paper III, were based on in vivo studies in an intra-individual approach, comparing surfaces/sites of these evaluated materials with resin composite and enamel. Paper I, II, and V are focused on dental biofilm formation and the gingival response. Paper III deals with the cytotoxic effects of the CAC, in vitro, and Paper IV is about the caries-associated microflora.

Subjects and surfaces of the in vivo evaluations

Paper I and II: Intra-individually comparisons were made regarding dental biofilm formation and initiation of gingivitis with and without oral hygiene in 15 subjects (4 females and 11 males, mean age 63 years, range 40-85) within a set of two cervical Class V restorations, one of CAC and one of resin composite, and one control surface of enamel. Twenty sets were included.

Paper IV: The study comprised two study groups. The first one corresponded to the study group described in Paper V. The levels of the mutans streptococci and lactobacilli in ten days old dental biofilm were compared between 11 of the 12 included approximal surfaces of leucite-reinforced bonded ceramic restorations (IPS Empress, Ivoclar, Liechtenstein), Class II restorations of resin composite and enamel. The second study group of subjects was in accordance with those described in Paper I. The levels of the caries-associated bacteria were compared between 18 cervical Class V restorations of CAC and resin composite and smooth non-restored enamel surfaces without detected caries.

Paper V: Intra-individually comparisons were made between one approximal surface of a bonded ceramic coverage (IPS Empress, Ivoclar, Liechtenstein) and one of enamel in 17 subjects (11 females and 6 males, mean age 54 years, range 43-85), regarding dental biofilm formation and initiation of gingivitis with and without oral hygiene. In a subgroup, including 11 of these subjects, an additional approximal surface of a Class II resin composite restoration (>1-year old) was also compared. Two sets
of the three surfaces; ceramic, resin composite and enamel, were compared within one subject. In all, 18 sets of ceramic and enamel were compared, 12 of which also included resin composite.

**Study Designs**

*Paper I and V:* The evaluations comprised a cross sectional study, investigating the effect of customary oral hygiene on dental biofilm removal and the gingival state adjacent to the included material and the enamel surfaces, and a ten-day experimental study in which the subjects refrained from oral hygiene to allow dental biofilm formation and subsequent initiation of gingivitis. Descriptive data of the subjects were recorded at the start of the study. The clinical parameters measured were intra-individually compared between the surfaces of CAC (*Paper I*) or leucite-reinforced, resin-bonded ceramic (*Paper V*), resin composite and enamel in the cross sectional study and on days 0, 3, 7, and 10 of the experimental gingivitis study (Fig. 1)

<table>
<thead>
<tr>
<th>Cross Sectional</th>
<th>Experimental gingivitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>1. GCF</td>
<td>GCF</td>
</tr>
<tr>
<td>2. GI ** GI</td>
<td>GI</td>
</tr>
<tr>
<td>3. GBI*</td>
<td>QHI</td>
</tr>
<tr>
<td>4. QHI</td>
<td>PI</td>
</tr>
<tr>
<td>5. PI*</td>
<td>SBI</td>
</tr>
<tr>
<td>6. SBI</td>
<td>PPD</td>
</tr>
<tr>
<td>7. PPD</td>
<td>DMFS*</td>
</tr>
<tr>
<td>8. DMFS*</td>
<td></td>
</tr>
</tbody>
</table>

*Fig 1.* Distribution of the clinical parameters included during the study period. Sampling of gingival crevicular flow (GCF), Gingival Index (GI, Löe 1967), gingival bleeding index (GBI; Ainamo and Bay 1975), modified Quigley-Hein’s plaque index (QHI; Turesky 1970), plaque index (PI; Lenox and Kopczyk 1973), sulcus bleeding index (SBI; Mühleman 1971), probing pocket depth (PPD). Descriptive variable of oral characteristics (*). The numbers indicate the chronological orders of measuring. Healthy gingiva (GI=0), gingival redness and swelling (GI=1) were estimated as the 2nd parameter and gingival bleeding (GI=2) was assessed after the plaque index (**).
Collection of GCF

The sites to be collected were isolated with cotton rolls and carefully sprayed with water to remove saliva. Each site was gently dried using an air syringe for ten seconds. A paper strip (Periopaper®) was inserted at the orifice of the gingival crevice and left there for 30 seconds (Paper I) or 10 seconds (Paper V). Fluid volumes were determined from standard curves using a Periotron 6000® (Paper I) or Periotron 8000® (Paper V) which were calibrated with certain volumes of human serum.

### Quigley Hein Plaque Index System

*(mod. by Turesky et al. 1971)*

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No plaque.</td>
</tr>
<tr>
<td>1</td>
<td>Separate flecks of plaque at the cervical margin of the tooth.</td>
</tr>
<tr>
<td>2</td>
<td>A thin continuous band of plaque (up to one mm) at the cervical margin of the tooth.</td>
</tr>
<tr>
<td>3</td>
<td>A band of plaque wider than one mm but covering less than one-third of the crown of the tooth.</td>
</tr>
<tr>
<td>4</td>
<td>Plaque covering at least one-third but less than two thirds of the crown of the tooth.</td>
</tr>
<tr>
<td>5</td>
<td>Plaque covering two-thirds or more of the crown.</td>
</tr>
</tbody>
</table>

**Paper II and V:** The levels of IL-1α, IL-1β and IL-1 ra in the sampled GCF from each site of CAC, resin composite and enamel in the cross sectional study and from days 0, 3, 7, *(Paper II)* and day 10 *(Paper V)* of the experimental gingivitis study, as described in *Paper I*, were analysed, calculated, and intra-individually compared.
Analysis of Interleukin-1

Immediately after sampling the GCF, the absorbing part of the strip was cut off and transferred to a coded plastic tube. The sample was eluted twice by adding 50 μl 0.9% sodium chloride and centrifuged at 3000 rpm and 5 °C for 20 min. The 100 μl supernatant was transferred to a tube containing 100 μl sodium chloride and stored frozen at 80˚ C. The levels of IL-1α, IL-1β and IL-1ra were quantified with Enzyme Linked Immunosorbet Assays (ELISAs) according to the instructions provided by the manufacturer. The supernatants from each individual set of the three sites were similarly diluted and run with the same assay. To calculate the levels of IL-1α, IL-1β and IL-1 ra in the GCF, the dilution factors and the collected volume were taken into account.

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal gingiva.</td>
</tr>
<tr>
<td>1</td>
<td>Mild inflammation. Slight change in color, slight oedema. <em>No bleeding on probing.</em></td>
</tr>
<tr>
<td>2</td>
<td>Moderate inflammation. Gingival redness and swelling and shiny gingival. <em>Bleeding on probing.</em></td>
</tr>
<tr>
<td>3</td>
<td>Severe inflammation. Marked redness and oedema. Ulceration. Tendency to spontaneous</td>
</tr>
</tbody>
</table>
Paper III: Specimens of three dental composites (QuiXfil, Tetric Ceram, Filtek Supreme), one zinc phosphate cement (Harvard Cement), one glass ionomer cement (Ketac Molar) and one CAC (DoxaDent), were prepared in triplicate, and sterilized with UV-radiation. PVC strips for ISO 10993-5 cytotoxicity test were used as positive and glass specimens as negative control. To be used fresh, half of the specimens was added to the cultures immediately after preparation and sterilization. The other half was preincubated at 37° C, pH 7.2 for seven days in cell culture medium. The culture medium was then removed and the specimens were used for the experiments. The experiments were repeated 6 times, which resulted in 6 observations per material (18 for fresh and 18 for 7 days old specimens).

Cytotoxicity determination

Murine L-929 fibroblasts (5 ml aliquots, containing 3x10^4 cells/ml), were cultivated in DMEM with 10 % FCS 1% glutamine and 1% penicillin/streptomycin at 37°C/5% CO₂ and exposed for the specimens for 72 h in polystyrene 6-well tissue culture plates. The cells were harvested with trypsin, centrifuged and resuspended in 500 µl DMEM. Cells were then counted in a volume of 500 µl DMEM over a fixed time of 30 s with a flow cytometer equipped with an argon laser tuned at 488 nm. Cell numbers after exposure to test specimens were compared to controls (cultures with glass specimens).

Paper IV: Ten days accumulated dental biofilm from the cervical surface of CAC or from the approximal surface of a resin-bonded, leucite-reinforced ceramic, and from the comparable surfaces of resin composite and enamel was collected after preceding water irrigation of the teeth. The plaque was sampled with a tip of a sterile applicator and immersed in 0.5 ml salt buffer (pH 7.2). Each sample was homogenized by pulsed ultrasonic oscillation and then diluted serially in the salt buffer. Aliquots of the samples were cultured on agar plates, which were incubated in 5% CO₂ and 95% air at 37° C for two days. Blood agar was used to determine the total counts of bacteria, Mitis salivarius agar supplemented with bacitracin, and Rogosa selective lactobacilli agar were used to estimate mutans streptococci and lactobacilli, respectively. The number of bacteria was counted as colony forming units (CFUs) and the relative number (% of total bacteria) was calculated.
Statistics

**Paper I**: The GCF volumes were analysed by paired t-test, and the Wilcoxon signed-rank test was used to show differences in plaque and gingival indices between the materials and enamel. The p-value was set at 0.05 to indicate statistical significance.

**Paper II**: The concentrations of IL-1α, IL-1β and IL-1 ra were analysed with Kolmogorov-Smirnov goodness of fit test for normality. Wilcoxon signed rank test was used for intra-individual comparisons between the materials and enamels. The p-value was set at 0.05 to indicate statistical significance.

**Paper III**: The data were analysed with Analysis of Variance and a post hoc test, developed by Ryan, Einot and Gabriel, and Welsch. The p-value was set at 0.05 and at 0.005 regarding multiple testing of the control.

**Paper IV**: The differences between the materials and enamel regarding the relative number of mutans streptococci and lactobacilli of the total bacterial counts were analysed with Wilcoxon signed-rank test and Exact test (Monte Carlo). The p-value was set at 0.05.

**Paper V**: The data were analysed with Kolmogorov-Smirnov goodness of fit test for normality. Wilcoxon signed rank test and Exact test (Monte Carlo) were used for intra-individual comparisons of plaque index, gingival index, GCF-volumes, and its concentrations of IL-1α, IL-1β and IL-1 ra. The p-value was set at 0.05.

In all papers, except for Paper III, the data were processed in SPSS (Statistical Package for Social Sciences, version 10.0 (Paper I-IV) and version 13.0 (Paper V). In Paper III, SAS© Release 8.2. was used for the statistical work.
**Results**

**Paper I:** In the cross-sectional study, plaque and gingival indices and the amounts of GCF did not differ between CAC, resin composite and enamel. An increase (p<0.05) of plaque and gingival indices was found between day 0 and day 10 of the experimental gingivitis study (Table 1). Neither the gingival index, nor the amounts of GCF differed between the CAC, resin composite and enamel on days 0 and 10. In the end of the experimental gingivitis, dental biofilm was present on all surfaces, but with a lower plaque index on surfaces of enamel than on surfaces of calcium aluminate cement (p=0.014) and resin composite (p=0.034).

**Table 1.** The means of the modified Quigley-Hein’s Plaque Index (QHI), the Gingival Index (GI), and the gingival crevicular fluid (GCF, μl/30s; standard deviation), adjacent to calcium aluminate cement (CAC), resin composite and enamel in the cross-sectional study (CSS) and on days 0, and 10 of the experimental gingivitis study. * QHI differed significantly between enamel compared with CAC (p=0.014) and resin composite. (p=0.034).

<table>
<thead>
<tr>
<th></th>
<th>CAC</th>
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<th>Composite</th>
<th>n</th>
<th>Enamel</th>
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</tr>
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<tbody>
<tr>
<td><strong>CSS</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QHI</td>
<td>0.5</td>
<td>20</td>
<td>0.4</td>
<td>20</td>
<td>0.4</td>
<td>20</td>
</tr>
<tr>
<td>GI</td>
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<td>20</td>
<td>0.2</td>
<td>20</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td>GCF</td>
<td>0.31 (0.31)</td>
<td>20</td>
<td>0.36 (0.42)</td>
<td>20</td>
<td>0.21 (0.23)</td>
<td>20</td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>QHI</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>GI</td>
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<td>0</td>
<td>18</td>
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<td>18</td>
</tr>
<tr>
<td>GCF</td>
<td>0.25 (0.30)</td>
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<td>0.28 (0.33)</td>
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<tr>
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<td>3.17*</td>
<td>18</td>
<td>2.83*</td>
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<tr>
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</tr>
<tr>
<td>GCF</td>
<td>0.43 (0.30)</td>
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<td>0.49 (0.47)</td>
<td>18</td>
<td>0.27 (0.25)</td>
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</table>
**Paper II:** The GCF concentrations of IL-1 α, IL-1 β and IL-1 ra in both the cross-sectional study and the experimental gingivitis study are given in Table 2. No significant differences were found regarding the IL-1 levels between the materials and enamel. The amount of the supernatants was inadequate to measure IL-1β on day 0. In the experimental gingivitis study, from day 0 to 7, IL-1 α levels increased adjacent to CAC (p=0.011), resin composite (p=0.041) and enamel (p=0.117). The enhanced levels of IL-1 ra were only significant adjacent to resin composite (p=0.002).

Table 2. The median concentration (range) of IL-1 α (ng/ml), IL-1 β (ng/ml) and IL-1 ra (µg/ml) in samples of gingival crevicular fluid adjacent to calcium aluminate cement (CAC), resin composite and enamel in the cross-sectional study (CSS) and on days 0, 3, and 7 of the experimental gingivitis study.

<table>
<thead>
<tr>
<th></th>
<th>CAC</th>
<th></th>
<th>Composite</th>
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<th>Enamel</th>
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<td></td>
<td>n</td>
<td></td>
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</tr>
<tr>
<td>IL-1 α</td>
<td>0</td>
<td>(0 – 64.8)</td>
<td>19</td>
<td>0</td>
<td>(0 – 30.2)</td>
<td>19</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>0</td>
<td>(0 – 17.8)</td>
<td>6</td>
<td>0</td>
<td>(0 – 7.9)</td>
<td>6</td>
</tr>
<tr>
<td>IL-1 ra</td>
<td>1.6</td>
<td>(0.1 – 8.7)</td>
<td>17</td>
<td>1.3</td>
<td>(0 – 4.8)</td>
<td>17</td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
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<td></td>
<td>n</td>
<td></td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>IL-1 α</td>
<td>0</td>
<td>(0 – 211.1)</td>
<td>16</td>
<td>0</td>
<td>(0 – 134.1)</td>
<td>16</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>IL-1 ra</td>
<td>0.8</td>
<td>(0 – 24.9)</td>
<td>15</td>
<td>1.0</td>
<td>(0 – 9.4)</td>
<td>15</td>
</tr>
<tr>
<td><strong>Day 3</strong></td>
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<td></td>
<td>n</td>
<td></td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>IL-1 α</td>
<td>9.8</td>
<td>(0 – 287.1)</td>
<td>16</td>
<td>5.8</td>
<td>(0 – 151.9)</td>
<td>16</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>0</td>
<td>(0 – 7.0)</td>
<td>15</td>
<td>0</td>
<td>(0 – 47.0)</td>
<td>15</td>
</tr>
<tr>
<td>IL-1 ra</td>
<td>2.6</td>
<td>(0.3 – 17.2)</td>
<td>15</td>
<td>1.6</td>
<td>(0.2 – 31.0)</td>
<td>15</td>
</tr>
<tr>
<td><strong>Day 7</strong></td>
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<td></td>
<td>n</td>
<td></td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>IL-1 α</td>
<td>28</td>
<td>(0 – 401.0)</td>
<td>16</td>
<td>9.1</td>
<td>(0 – 153.9)</td>
<td>16</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>0</td>
<td>(0 – 12.1)</td>
<td>15</td>
<td>0</td>
<td>(0 – 25.4)</td>
<td>15</td>
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<tr>
<td>IL-1 ra</td>
<td>6.0</td>
<td>(1.2 – 22.2)</td>
<td>15</td>
<td>8.6</td>
<td>(2.8 – 33.7)</td>
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</tr>
</tbody>
</table>
**Paper III:** The six dental direct restorative materials showed different influences on L-929 fibroblasts cytotoxicity \( p<0.0001 \). The cytotoxicity of all specimens diminished with increasing preincubation time \( p < 0.0001 \) but in various degree \( p<0.0001 \). The validity was checked by a positive control, which reduced cell numbers to 18.7 (±9.8) % of negative control as expected from previous experiments. All freshly prepared materials showed reduced cell numbers compared to the negative control. A rank order of significantly different cytotoxic effects was established for fresh (Fig. 2a) and 7 days preincubated specimens (Fig. 2b). Fresh specimens of DoxaDent exhibited the lowest cytotoxicity, followed by QuiXfil and a group of three materials (Tetric Ceram, Filtek Supreme, Harvard Cement). Ketac Molar showed the highest cytotoxicity (Fig 2a). As shown in Fig 2b, after 7 days of preincubation, Harvard Cement and Filtek Supreme demonstrated more cytotoxicity than the other materials \( p < 0.005 \).

Fig. 2a.
**Fig. 2b.**

Grouping of materials with no different effects: (a) freshly prepared, (b) 7 day preincubated. Specimens were incubated with L-929 fibroblasts for 72 h and cell numbers determined by flow cytometry. Cell numbers were expressed as a percentage of controls (cultures with glass specimens). Vertical bars show 1 second-means ± standard deviations of 18 observations (= 6 independent experiments with triplicates); materials covered with the same horizontal bar are not significantly different from each other. Stars indicate statistical significant difference to control (=100%): *= p<0.05, ** = p<0.01, ***p<0.005 (Because of multiple testing a p-value < 0.005 was considered to indicate statistical significance for this special question).
**Paper IV:** The levels of mutans streptococci and lactobacilli in approximal plaque on leucite-reinforced bonded ceramic, resin composite and enamel did not differ (Table 3 a). Nor, were there any differences found between the levels of caries-associated bacteria on smooth surfaces of CAC, resin composite and enamel (Table 3 b).

Table 3. Caries-associated bacteria, *in vivo*, on leucite-reinforced ceramic (a), CAC (b), resin composite and enamel. The relative proportions (percentage of total bacteria) of mutans streptococci and lactobacilli in 10 days accumulated dental biofilm on a) approximal surfaces of leucite-reinforced ceramic, resin composite, and enamel (n=11), and b) smooth surfaces of CAC, resin composite, and enamel (n=17).

<table>
<thead>
<tr>
<th></th>
<th>Empress n=11</th>
<th>Resin composite n=11</th>
<th>Enamel n=11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>relative numbers of the total bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0% &gt;0-1% &gt;1%</td>
<td>0% &gt;0-1% &gt;1%</td>
<td>0% &gt;0-1% &gt;1%</td>
</tr>
<tr>
<td>mutans streptococci</td>
<td>5 2 4</td>
<td>4 5 2</td>
<td>4 7 0</td>
</tr>
<tr>
<td>lactobacilli</td>
<td>6 4 1</td>
<td>9 2 0</td>
<td>11 0 0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>CAC n=17</th>
<th>Resin composite n=17</th>
<th>Enamel n=17</th>
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<tbody>
<tr>
<td><strong>relative numbers of the total bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0% &gt;0-1% &gt;1%</td>
<td>0% &gt;0-1% &gt;1%</td>
<td>0% &gt;0-1% &gt;1%</td>
</tr>
<tr>
<td>mutans streptococci</td>
<td>2 11 4</td>
<td>1 13 3</td>
<td>1 13 3</td>
</tr>
<tr>
<td>lactobacilli</td>
<td>12 5 0</td>
<td>12 5 0</td>
<td>9 6 2</td>
</tr>
</tbody>
</table>
**Paper V:** Plaque and gingival indices did not differ between approximal surfaces of bonded ceramic, resin composite and enamel in the cross-sectional study and on days 0 and 10 of the experimental gingivitis study. Sites adjacent to ceramic surfaces showed a significantly higher amount of GCF compared with enamel in both the cross sectional study \((p=0.017)\), and day 0 \((p=0.019)\) and day 10 of the experimental gingivitis study \((p=0.016)\). Each of these parameters increased significantly between day 0 and 10 of the experimental gingivitis study at sites of ceramic restorations \((p<0.001)\), resin composite restorations \((p<0.009)\) and enamel control surfaces \((p<0.002)\). Descriptive data are shown in Table 4.

The levels of IL-1\(\alpha\), IL-1\(\beta\) and IL-1\(\text{RA}\) in the GCF did not differ between the ceramic, resin composite and enamel surfaces neither in the cross-sectional study, nor on days 0 and 10 of the experimental gingivitis study. IL-1\(\alpha\) increased at ceramic sites \((p=0.024)\), resin composite sites \((p=0.034)\) and enamel sites \((p=0.028)\) from day 0 to day 10 of the experimental gingivitis. IL-1\(\text{RA}\) also increased at the resin composite sites \((p=0.027)\). Descriptive data are shown in Table 5.
Table 4. The means of the modified Quigley-Hein Plaque Index (QHI), Gingival Index (GI), and gingival crevicular fluid (GCF; μl/10s; standard deviation) adjacent to approximal surfaces of a leucite-reinforced ceramic, resin composite and enamel in the cross sectional study (CSS) and on days 0 and 10 of the experimental gingivitis study. n = number of sets of the intra-individually compared surfaces of ceramic and enamel (n=18), also including the evaluated composite subgroup (n=12).

<table>
<thead>
<tr>
<th></th>
<th>Ceramic</th>
<th>Composite</th>
<th>Enamel</th>
<th>n</th>
</tr>
</thead>
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<tr>
<td><strong>CSS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QHI</td>
<td>0.94</td>
<td>-</td>
<td>1.06</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>1.1</td>
<td>0.83</td>
<td>1.4</td>
<td>12</td>
</tr>
<tr>
<td>GI</td>
<td>0.61</td>
<td>-</td>
<td>0.28</td>
<td>18</td>
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<tr>
<td></td>
<td>0.75</td>
<td>0.5</td>
<td>0.42</td>
<td>12</td>
</tr>
<tr>
<td>GCF</td>
<td>0.49 (0.10) *</td>
<td>-</td>
<td>0.22 (0.23) *</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>0.49 (0.41)</td>
<td>0.32 (0.36)</td>
<td>0.24 (0.23)</td>
<td>12</td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QHI</td>
<td>0.33</td>
<td>-</td>
<td>0.43</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>12</td>
</tr>
<tr>
<td>GI</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>GCF</td>
<td>0.30 (0.33) *</td>
<td>-</td>
<td>0.13 (0.19) *</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>0.23 (0.25)</td>
<td>0.18 (0.26)</td>
<td>0.19 (0.16)</td>
<td>12</td>
</tr>
<tr>
<td><strong>Day 10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QHI</td>
<td>2.62</td>
<td>-</td>
<td>3.06</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>2.67</td>
<td>3.33</td>
<td>3.17</td>
<td>12</td>
</tr>
<tr>
<td>GI</td>
<td>1.00</td>
<td>-</td>
<td>0.93</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.08</td>
<td>0.83</td>
<td>12</td>
</tr>
<tr>
<td>GCF</td>
<td>0.82 (0.41) *</td>
<td>-</td>
<td>0.49 (0.38) *</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>0.78 (0.34)</td>
<td>0.51 (0.54)</td>
<td>0.44 (0.37)</td>
<td>12</td>
</tr>
</tbody>
</table>

* p< 0.05
Table 5. The median concentration (range) of interleukin (IL) -1 α (ng/ml), IL -1 β (ng/ml) and IL-1 receptor antagonist (ra; µg/ml) in samples of GCF adjacent to ceramic, resin composite and enamel in the cross-sectional study (CSS) and on days 0 and 10 of the experimental gingivitis study. The digit zero (0) represents all values below the detection levels (IL-1α, IL-1 β, < 1 pg/ml; IL-1 ra < 14 pg/ml) of the assays. n = number of sets of the intra-individually compared surfaces of ceramic and enamel, also including the evaluated composite subgroup.

<table>
<thead>
<tr>
<th></th>
<th>Ceramic</th>
<th>Composite</th>
<th>Enamel</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CSS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1 α</td>
<td>15.0 (0 - 64.6)</td>
<td>-</td>
<td>22.5 (0 - 98.3)</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>15.6 (0 - 64.6)</td>
<td>2.7 (0 - 273.0)</td>
<td>57.8 (0 - 98.3)</td>
<td>11</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>17.2 (0 - 117.4)</td>
<td>-</td>
<td>22.6 (0-1088.7)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>9.5 (0 - 55.4)</td>
<td>2.6 (0 - 154.9)</td>
<td>27.8 (0 - 123.0)</td>
<td>9</td>
</tr>
<tr>
<td>IL-1 ra</td>
<td>1.3 (0 - 6.7)</td>
<td>-</td>
<td>2.1 (0 - 24.3)</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>1.3 (0 - 6.7)</td>
<td>1.4 (0 - 14.3)</td>
<td>2.4 (0 - 24.3)</td>
<td>11</td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1 α</td>
<td>1.0 (0 - 49.3)</td>
<td>-</td>
<td>0 (0 - 60.5)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>2.5 (0 - 49.3)</td>
<td>0 (0 - 131.6)</td>
<td>0 (0 - 60.5)</td>
<td>12</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>0.7 (0 - 33.0)</td>
<td>-</td>
<td>0 (0 - 297.5)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>0 (0 - 29.5)</td>
<td>4.9 (0 - 62.5)</td>
<td>0 (0 - 17.5)</td>
<td>9</td>
</tr>
<tr>
<td>IL-1 ra</td>
<td>0.8 (0 - 5.0)</td>
<td>-</td>
<td>1.2 (0 - 6.3)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1.3 (0 - 5.0)</td>
<td>0.7 (0 - 3.1)</td>
<td>1.0 (0 - 6.3)</td>
<td>10</td>
</tr>
<tr>
<td><strong>Day 10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1 α</td>
<td>13.5 (0 - 113.9)</td>
<td>-</td>
<td>10.8 (0 - 380.6)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>19.4 (1.0 - 113.9)</td>
<td>17.3 (0 - 109.0)</td>
<td>10.8 (0.9 - 102.0)</td>
<td>12</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>7.4 (0 - 63.7)</td>
<td>-</td>
<td>6.5 (0 - 206.7)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>8.8 (0 - 63.7)</td>
<td>15.6 (5.4 – 89.6)</td>
<td>2.5 (0 - 145.2)</td>
<td>9</td>
</tr>
<tr>
<td>IL-1 ra</td>
<td>1.4 (0 - 5.7)</td>
<td>-</td>
<td>2.2 (0 - 58.0)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1.8 (0.1- 5.7)</td>
<td>3.4 (0.5 – 7.1)</td>
<td>5.4 (0 - 15.6)</td>
<td>10</td>
</tr>
</tbody>
</table>
Discussion

The discussion of this thesis focuses on the influence of ceramic restorative surfaces on dental biofilm formation, subsequent gingival response and on the caries-associated microflora in vivo. Although discussed in the same context, leucite-reinforced ceramic and CAC are totally different types of ceramic materials with regard to their contents, manufacturing, handling properties and indications for use. The evaluated ceramic surfaces were compared with resin composite, as a reference material, and with enamel, as a control, which are both involved in the discussion. Influence of dental materials on biofilm and gingiva is only a part of their biocompatibility. At development of a new, alternative restorative material, its fundamental biocompatibility on a cellular level should be evaluated. Therefore, even the cytotoxicity of the CAC on fibroblasts is discussed briefly.

Biofilm formation on CAC (Paper I)

In the in vivo studies described in Paper I and II, an experimental version of the CAC in the oral environment on smooth, mainly buccal, tooth surfaces was evaluated. Restorations of both CAC and resin composite assembled higher amounts of biofilm than enamel after ten days of neglected oral hygiene (Paper I). In this way the CAC does not seem to be a superior alternative to resin composite. The clinical relevance of the differences of the dental biofilm amounts can be considered as small, but not necessarily scanty in individuals with poor oral hygiene and many restorations. Still, among individuals with good to moderate oral hygiene, the buccal/lingual surfaces seem to be possible to clean satisfactorily with current oral hygiene irrespective of the underlying surface material. The initial bacterial adhesion is dependent on the physiochemical properties of the pellicle, which can be influenced by the nature of the underlying hard surface (Teughels, 2006). Dental biofilm formation in the first hours can be affected by the surface energy (Quirynen 1995). However, after six hours of pellicle formation surface, differences between various dental materials seem to be camouflaged (Hannig 1997) and a similar biofilm forms within 24 hours (Hannig 1999). After several days of dental biofilm formation, differences in the amounts of dental
biofilm could rather be explained by the roughness of the underlying surface. Increased surface roughness or porosities and voids of the intra-oral hard surfaces can facilitate biofilm formation, probably because of a larger surface area or by affecting the bacterial retention. Dental biofilm amounts in the defects might function as “plaque reservoirs”, which can enhance the biofilm formation (Quirynen 1989, 1995). The range in surface roughness of different hard surfaces is wide (Bollen et al. 1997). CAC has a heterogenous structure with porosities (Geirsson et al. 2004 b) which makes it difficult to obtain a surface with low surface roughness, without chips, pits and grooves, even after finishing (Sunnegård-Grönberg and van Dijken 2003), which might have contributed to the enhancement of biofilm formation after ceased oral hygiene.

To summarize, as regards the biofilm formation on Class V restorations of CAC, this material can be considered as an acceptable one in parity with resin composite. Without a proper oral hygiene, it could be valuable to pay attention to possible accumulations of biofilm on available restorative surfaces of CAC, as well as on surfaces of resin composite.

*Biofilm formation on bonded ceramic (Paper V)*

With or without oral hygiene, similar amounts of biofilm were present on the approximal surfaces of the bonded ceramic coverages, as compared to enamel. That observation is in accordance with clinical follow-ups of leucite-reinforced ceramic restorations (Sjögren et al. 1999, Stenberg and Matsson 1993, Tidehag and Gunne 1995). Nevertheless, higher GCF amounts were found adjacent to the bonded ceramic than adjacent to enamel (*Paper V*). As discussed in this paper, there could be a possible effect of the misallocation of the included restorations. Most ceramic coverages were placed in molars as compared to resin composite and enamel surfaces, which were both mainly located in premolars. Molars have wider approximal contacts, not accessible to ocular surveys. Therefore, it could be theorized, that the increased amounts of the GCF were evoked by a response to “hidden” biofilms on the approximal surfaces. Another reason for this response may be non-detected overhangs formed by resin composite luting agents (O’Rourke et al. 1995) and marginal deficits, which can be present irrespectively of the luting cement used (Krämer and Frankenberger 2005). Overhanging margins can
result in enhanced plaque and gingival indices (Lang et al. 1988). Observations have been made of a slight overextension just above the cervical margin of 1-2 years old leucite-reinforced ceramic crowns with subgingival finish lines (Gemalmaz and Ergin 2002) and of approximal marginal excess of luting cement on radiographs of ceramic inlays (Pallesen and Dijken, van 2000).

The degradation of dental ceramics generally occurs because of mechanical forces or chemical attack. Nevertheless, the chemical durability of dental ceramic is excellent, except for excessive exposure to acidulated fluoride (Anusavice 1992). To put the light of what is known about ceramics and dental biofilm formation, even other ceramics than the evaluated leucite-reinforced ceramic is involved in the discussion. The dental biofilm formation in vivo on glazed glass ceramics and shaded sintered ceramics seems to be similar (Castellani et al. 1996, Hahn et al. 1993). In contrast, Hahn et al. (1992) found that biofilm formation differs between glazed and polished ceramics of various bulk materials.

Over the years, plaque index, bleeding on probing and pocket depth adjacent to inlays were increasing. Marked wear of ceramic inlays has been observed at aging. In addition, the surface roughness might increase because of grinding or finishing at luting (Pallesen and Dijken, van 2000). With the exception of glazed surfaces, the surface roughness of ceramic materials can affect the biofilm formation (Kawai et al. 2000). Recently, preliminary results show a larger tendency to accumulate biofilm on glazed zirconium ceramics than on the polished ones (Scotti et al. 2006). Less dental biofilm formation on glazed ceramic surfaces than on non-glazed ceramic surfaces has been reported (Castellani et al. 1996). However, marginal defects and wear of the visible part of the approximal surfaces were only found in a minority of the current restorations in the clinical follow-up (Dijken van et al. 2001).

Evaluations of biofilm formation on ceramics in comparison with resin composite are sparsely reported. In vitro, lower amounts of bacteria and glucans adhere to surfaces of ceramic than to surfaces of resin composite (Kawai and Urano 2001). It has been observed that the total counts of the facultative bacteria do not differ between buccal surfaces of ceramic and restorative composite in situ (Tanner et al. 2005). In Paper V, a similar
biofilm formation on approximal surfaces of ceramic and resin composite restorations was noted.

The dental biofilm formation can be favoured at gingivitis (Quirynen 1991), probably because of an increased supply of the nutrient GCF (Marsh 2003). Despite the higher amounts of GCF adjacent to the bonded ceramic surfaces as compared to enamel, the dental biofilm formation on the visible part of the surface was not enhanced (Paper V). Although the difference in the supply of GCF can be considered as small, this might possibly support the observations of a retarded biofilm formation in vivo on ceramic restorations compared to enamel (Hahn et al. 1993) or contralateral teeth (Savitt et al. 1987, Jensen et al. 1990). If so, this advantage of the ceramic material is probably counteracted by side-effects such as aging/abrasion and luting/bonding, since lower amounts of dental biofilm on the bonded ceramic coverages than on enamel failed to be shown.

Thus, resin-bonded ceramic coverages were not shown to disfavour the biofilm formation, which implicates that the choice of leucite-reinforced ceramics as a restorative material should rely on other factors. The need for accurate oral hygiene on this material is not reduced as compared to that on resin composite and enamel.

The gingival response at sites of CAC and the bonded ceramic (Paper I, II, V)

The higher biofilm amounts on the surfaces of CAC and of resin composite than on enamel did not result in corresponding higher amounts of GCF (Paper I) and its levels of IL-1α, IL-1β and IL-1ra (Paper II). However, in the end of the biofilm formation period, a non-significant tendency towards higher crevicular fluid amounts adjacent to the CAC and resin composite was observed as compared to the remaining low volumes adjacent to the enamel (Paper I). The higher amounts of GCF at bonded ceramic sites than at enamel sites were not reflected in higher levels of IL-1α, IL-1β and IL-1ra (Paper V). There are no available reports in vivo as concerns the levels of proinflammatory cytokines in GCF adjacent to dental materials. Lipopolysaccharides, which can initiate a variety of biological responses, including IL-1, have been shown to have an affinity for resins, alloys, and even dental ceramics, which could contribute to an inflammatory process.
(Robinson et al. 1996). However, no significant influence of the ceramic specimens on the response of IL-1β release from gingival fibroblasts has been reported in vitro (Ozen et al. 2005).

Restorative surfaces below the gingival margin are associated with gingivitis (Schä tzle et al. 2000), ceramic restorations too (Gemalmaz and Erging 2002, Reitemeier et al. 2002). The cervical margin of the current evaluated sub-gingival fillings was not deep extended in an apical direction. The clinical expressions of gingivitis seem to be mainly low, and not influenced by the presence of the bonded ceramic and CAC (Paper I and V). This is also supported by the fact that a low gingival index was observed both at baseline and after four years for glass ceramic crowns (Kelsey et al. 1995). That is satisfactory, since gingivitis is a prerequisite of developing periodontitis (Kinane et al. 2006), and, in elder people considerably more attachment loss was found at sites with bleeding on probing (Gingival Index 2) than at sites with a Gingival Index 0 (Schä tzle et al. 2003).

As a whole, the clinical gingival health and the levels of IL-1 do not seem to be affected by the CAC restorations or the resin-bonded ceramic coverages, neither at current oral hygiene, nor at initiation of gingivitis.

Secondary findings of dental biofilm formation and gingival response (Paper I, II, V)

A few issues, not primarily associated with the general aims, will be discussed, that is, the general findings over the experimental gingivitis period, and the discussion as concerns the reference material of resin composite.

Dental biofilm formation during experimental gingivitis has shown to be followed by an enhancement of GCF (Zhang et al. 2002) without respect to resin composite and enamel (Dijken van et al. Sjöström 1998). The increase in GCF was also obvious in the study of the approximal surfaces (Paper V), but not significant at the buccal/lingual surfaces evaluated in Paper I. The levels of IL-1α were enhanced during the experimental gingivitis (Paper II and Paper V), which has also been observed previously (Kinane et al 1992). An increase in IL-β during experimental gingivitis has been shown (Deinzer et al. 1999, Gonzales et al. 2001, Zhang et al. 2002).
Nevertheless, a significant increase in IL-1β failed to be demonstrated, to a part, probably because of a low number of sites analyzed (Paper II). Although Wilton et al. (1992) could not correlate IL-1 levels with the plaque and gingival index, Hou et al. (1995) observed a considerable increase in the amount of IL-1β at bleeding sites compared to sites with a Gingival Index < 2. No gingival bleeding could be applied to most of the current evaluated sites (Paper I and V). IL-1α (Rasmussen et al. 2000) and IL-1β (Boström et al. 2000, Gamonal et al. 2000, Hou et al. 1995, Orozco et al. 2006) are associated with inflammation on a periodontitis level but not always detected in the control group of clinically healthy individuals (Gamonal et al. 2000, Wilton et al. 1992). There are contradictory findings regarding the levels of IL-1ra in GCF, at healthy versus inflammatory sites. Higher levels of IL-1 ra have been found at periodontitis sites than at non-inflamed gingival sites (Boström et al. 2000, Ishihara 1997) in divergence to Rawlinson et al. (2000). Experimental gingivitis does not seem to exert effects on the levels of IL-1 ra (Waschul et al. 2003). Among the current findings, increase in IL-ra was only found adjacent to Class V restorations of resin composite (Paper II), but the enhancement was not present at approximal sites (Paper V). The levels of IL-1 ra in GCF were approximately 1000-fold that of IL-1, which is in accordance with Boström et al. (2000). Other cytokines may also contribute to the balance between the production of IL-1 and of IL-1 ra in disease. For example, IL-4 TGF beta and IL-10 increase the production of IL-1 ra but at the same time decrease the production of IL-1 (Dinarello and Wolff 1993).

The higher amounts of dental biofilm, on smooth, cervical surfaces of resin composite as compared to enamel, (Paper I) were not demonstrated on approximal surfaces (Paper V). It is possible, that the approximal surfaces of resin composite compared to the buccal or lingual ones are less exposed for wear and roughening from abrasion via tooth-brushing, which preserves a relatively smooth surface (Folwaczny et al. 2000). When comparing the evaluations in these papers (Paper I and V), one should bear in mind that these studies did not take into consideration the individual variations caused by contributing factors, such as smoking. Aging of resin composite restorations can result in increased dental biofilm retention and degraded surfaces which may favour bacterial recolonization and complicate dental biofilm removal (Dijken van et al. 1985, Dijken van et al. 1987a). On early dental composites, dental biofilm forms more easily than on enamel (Larato 1972). Besides, subgingival resin composite restorations may have some negative effects on the quantity and quality of the plaque, increasing the anaerobic microflora...
(Paolantonio et al. 2004). Several studies did not reveal any differences in dental biofilm formation (Hahn et al. 1993) or subsequent clinical signs of gingivitis between sites of resin composites and enamel (Dijken van et al. 1987 a, Dijken van et al. 1987 b, Dijken van and Sjöström 1991 b, Dijken van and Sjöström 1998). Nor were there any differences of the gingival response observed between the sites of resin composite and enamel (Paper V). In contrast, less amounts of GCF have been collected at sites of enamel than at sites of resin composite restorations (Dijken van et al. 1987 a, Dijken van et al. 1987 b, Dijken van and Sjöström 1991 b).

Caries-associated bacteria on CAC and bonded ceramic (Paper IV)

Strategies to control caries include the inhibition of biofilm development such as prevention of attachment of cariogenic bacteria, or enhancement of the host defences (Marsh 2006). The proportions of mutans streptococci and lactobacilli in approximal dental biofilm on CAC and on aged, resin-bonded ceramics resembled those on resin composite and enamel. These materials did not influence the caries-associated microflora (Paper IV). The most frequent proportions of mutans streptococci and lactobacilli observed in this thesis were below 1%, irrespective of the underlying material. In vivo, the counts of mutans streptococci have not been showed to be different on buccal surfaces of ceramic and of restorative composites (Tanner et al. 2005). In vitro, a normal oral flora has been able to colonize resin materials (Suljak et al. 1995), which in cultures, however, might have a growth-enhancing effect on mutans streptococci (Friedl et al. 1992) and on lactobacilli (Hansel et al. 1998).

The lack of influence of the bonded ceramic on the caries-associated flora is not surprising, since ceramics are considered to be chemically stable. As discussed in Berglund et al. (2006), a depletion of calcium (Ca) and an increase in other elements (Al, Si, and Zr) have been found on the surface of wet specimens of CAC. However, the leakage of components from the CAC has not been extensively investigated.
The cytotoxicity and biocompatibility of the CAC (Paper III)

The CAC was developed in the end of the nineties as a “bioceramic” restorative material, aimed to be an alternative to the current filling materials which might have a cytotoxic potential (Ferracane 1994, Wrangsjö 2001). The cytotoxicity of the CAC on fibroblasts was lower than that of various resin composites as concerns fresh filling materials (Paper III). The disparity decreased in time. After one week, only one resin composite material (Filtek Supreme) and the phosphate cement, which is used as a temporary material, demonstrated significantly more cytotoxic effects than the CAC. However, the CAC was the only material which did not differ from the control. In this way, the CAC seems to be highly biocompatible.

At present, the use of the CAC as a restorative material is contra-indicated due to the deficient dimensional stability over time (Berglund et al. 2006), inferior mechanical properties (Geirsson et al. 2004 a, Sunnegård-Grönberg 2004) and short clinical durability (Sunnegård-Grönberg 2004). However, attempts have been made to use CAC, slightly modified, as a biomaterial in the other parts of the body, such as bone cement (Oh et al. 2002).

Thus, in vitro, fresh CAC exhibited the lowest cytotoxicity compared with other current dental materials. All materials showed diminished cytotoxicity over time. Although slightly cytotoxic, CAC can be considered as a biomaterial, which suggests further development to overcome its low mechanical properties.

General discussion of the methods

The studies (Paper I, II, IV, and V) were performed in an intra-individual approach, comparing surfaces within each individual, and ruling out possible host and life-style factors, which could have an impact on the results. For example, inter-individual variations are observed as concerns rate of dental biofilm formation and the development of gingivitis (Simonsson et al. 1987, Trombelli 2004) and the genotypes of IL-1 (Shapira 2005). Furthermore, GCF flow and IL-1α levels (Kinane and Chestnut 2000), gingival index and bleeding scores (Preber and Bergström, 1986, Lie
et al. 1998) are reduced in heavy smokers. The intra-individual study design decrease the number of participants needed for inclusion.

Efforts were made to compare similar surfaces fulfilling the inclusion criteria. According to the ecological plaque hypothesis (Marsh 2004), the local environment is not identical with other sites in the oral cavity. There are variations in the biofilm composition of smooth surfaces, fissures, gingival sulcus, in sites close to saliva excretion, and in sites differing in redox potential or nutrient availability (Arweiler et al. 2004). Besides, there is an impact of the intra-oral location on the rate of biofilm growth (Furuichi et al. 1992, Quirynen et al. 1991). However, the thickness of the buccal biofilm does not seem to be related to the location in the oral cavity (Auschill et al. 2004). With oral hygiene, a relatively good symmetrical plaque pattern has been observed, however, the plaque per cent of the tooth area differed between the adjacent first and second molars (Söder et al. 2003).

Alternative methods to evaluate restorative materials in vivo, are the use of attached splints, such as in studies of Hahn et al. (1992), or with temporarily bonded specimens (Tanner et al. 2005), which make it possible to evaluate different restorative materials at the same site or location. However, these methods did not, in full, mirror the clinical situation and the influences of ageing of the restorations. Furthermore, the local oral environment can be changed in course of time due to alterations of life-style related factors.

The development of gingivitis is associated with the quantity of the dental biofilm (Löe et al. 1965, Lie et al. 1998). In both Paper I and V, the plaque index used measures the extent of the dental biofilm covering the tooth based on a scale of six steps (Turesky et al. 1970) and quantification indices are considered to be necessary in clinical trials (Galgut 1999). An interval-scaled plaque index, developed for approximal surfaces (Matthijs et al. 2001, Dombret et al. 2003), might have been more appropriate to utilize in the study of bonded ceramics (Paper V). However, that index was not well-established at the time for measuring. The thickness and alterations of the quality of the dental biofilm during experimental gingivitis were not evaluated in the involved studies (Paper I and V). Nevertheless, a weak or strong inflammatory response to experimental
gingivitis is not caused by major differences in the composition of the oral microbiota (Lie et al. 1998).

The length of the experimental gingivitis period was based on previous studies (van Dijken et al. 1987a, 1987b, Dijken van and Sjöström 1991b, Dijken van and Sjöström 1998). If requested, the opposite arch was polished after measuring to support the compliance. That might have decreased the rate of dental biofilm formation with a subsequent delay of the development of gingivitis (Quirynen 1989). Moreover, there are differences in the gingival response to biofilm between low- and high-responders (Trombelli 2004). The distribution of low- and high responders in the study group is not known. However, at the start of the studies, the full mouth recorded gingival index was mainly low, as compared to the plaque index. The benefit of a further length of the experimental gingivitis period can be discussed; however, the primary aim was to compare the ceramic material under the prevailing conditions at initiation of gingivitis, not to evaluate the development of gingivitis per se.

The gingival index (Löe and Silness 1965, Löe 1967) is probably the most widely used index in clinical trials (Ciancio 1996). The determination of the volumes of GCF with Periotron® is a reliable method (Bickel and Cimasoni 1984), at least at volumes above 0, 2 µl (Chapple et al. 1995) and at appropriate calibration and handling of the apparatus (Ciantar and Carauna 1998). Presence of biofilm at collection of GCF can elevate the measured levels (Stoller et al. 1990), but later it is reported that the validity of the measurements was not affected by the presence of supragingival dental biofilm (Deinzer et al. 2000). It is necessary to distinguish between the initial collected volume of GCF, and its flow rate, of which the latter could be associated to the Gingival Index and can be a preferable method (Griffiths et al. 1992). Various tooth types associated with different sulcus depth or sampling areas might also have an impact on the sampling of the fluid. However, at collection of the fluid, there was a great attempt to insert the paper strips until the same depth of the sulcus. Besides, there is a risk of stimulating the gingival flow at repeated measures. Not to surpass the potential collection volume of the paper strip, the time for collection of the GCF was determined in a pilot study, that is, 30 s for buccal/lingual sites (Paper I) and 10 s for approximal sites (Paper V). The protein recovery from various paper types used to collect GCF can vary, and low protein concentrations are less efficiently eluted.
from the paper strips than high protein concentrations. Although another type of collecting papers might be more efficient for protein recovery (Johnson et al. 1999), the used paper strips were adapted to measure the fluid volume with Periotron® and the papers have been used previously for sampling of IL-1 (Deinzer et al. 1999, Waschul et al. 2003). It has to be noted that the measures of the GCF are related to be surrogate measures, such as the Gingival Index or other measures of gingival inflammation (Socransky and Haffajee 2005).

Conventional methods of culturing caries-associated bacteria on selective media and in an aerobic environment supplemented with carbon dioxide were used (**Paper IV**). It could be discussed, if culturing the lactobacilli under anaerobic conditions would be more appropriate for optimal growth. Though almost all lactobacilli are facultatively anaerobic bacteria and thus grow both under aerobic and anaerobic conditions, it is known that the growth is enhanced in an anaerobic or a microaerophilic environment. However, optimizing growth conditions not necessarily mean that a higher number of lactobacilli is detected. A pilot study further supported this assumption. Thus, the outcome of lactobacilli detection hardly would have been different if an anaerobic incubation had been the first choice.

More sensitive or sophisticated tests can also be used, such as molecular biology tests to characterize the quality of the biofilm. In **Paper III**, the applied method for cytotoxicity testing of the dental materials was cell counting by flow cytometry (Schedle et al.1998, Franz et al. 2003) comprising cells which were not lyzed by apoptosis and/or necrosis. At flow cytometry, the analysis is performed of a large numbers of cells at a single-cell level. The measurements are quantitative and, in addition, many cell parameters can be evaluated simultaneously. Cytotoxicity tests can also rely on tests of the cell viability measuring cellular metabolic activity and functional state of the mitochondria such as in Lönnroth et al. (2001). Nevertheless, to be used as preliminary and as initial tests on the influence of the restorative materials in comparison to other materials or controls, the test method used is established and makes the study comparable to previous studies evaluating restorative materials (Schedle et al. 1998, Franz et al. 2003).

With the limitations of the methods discussed in mind, the results should be interpreted carefully.
From studies in this thesis, recurrent findings of “no significant differences between the evaluated materials and enamel” are reported. The bias against publishing ‘negative’ results has a tremendous impact on the literature, and recognition of the importance of such results is needed (Kinane et al. 2005).

By nature, this thesis is focused on a limited part of the influences of restorative materials on the oral health. One has to bear in mind the importance of applying any side-effects of the restorative material in a holistic approach to the oral environment and to the patient, which is after all necessary to establish and maintain a good oral health.
Conclusions

The main conclusions from the findings of this thesis are as follows:

The low cytotoxic effect of CAC and the limited increase in dental biofilm formation on that material compared with enamel suggest CAC to be a biocompatible dental material with respect to dental biofilm formation, presence of caries-associated microflora and gingival response. This finding, together with the observation that the influence of bonded ceramic on dental biofilm formation, caries-associated microflora and clinical gingivitis was not different from that of enamel, implicates for both CAC restorations and bonded ceramic that the need of oral hygiene is not reduced.
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References


