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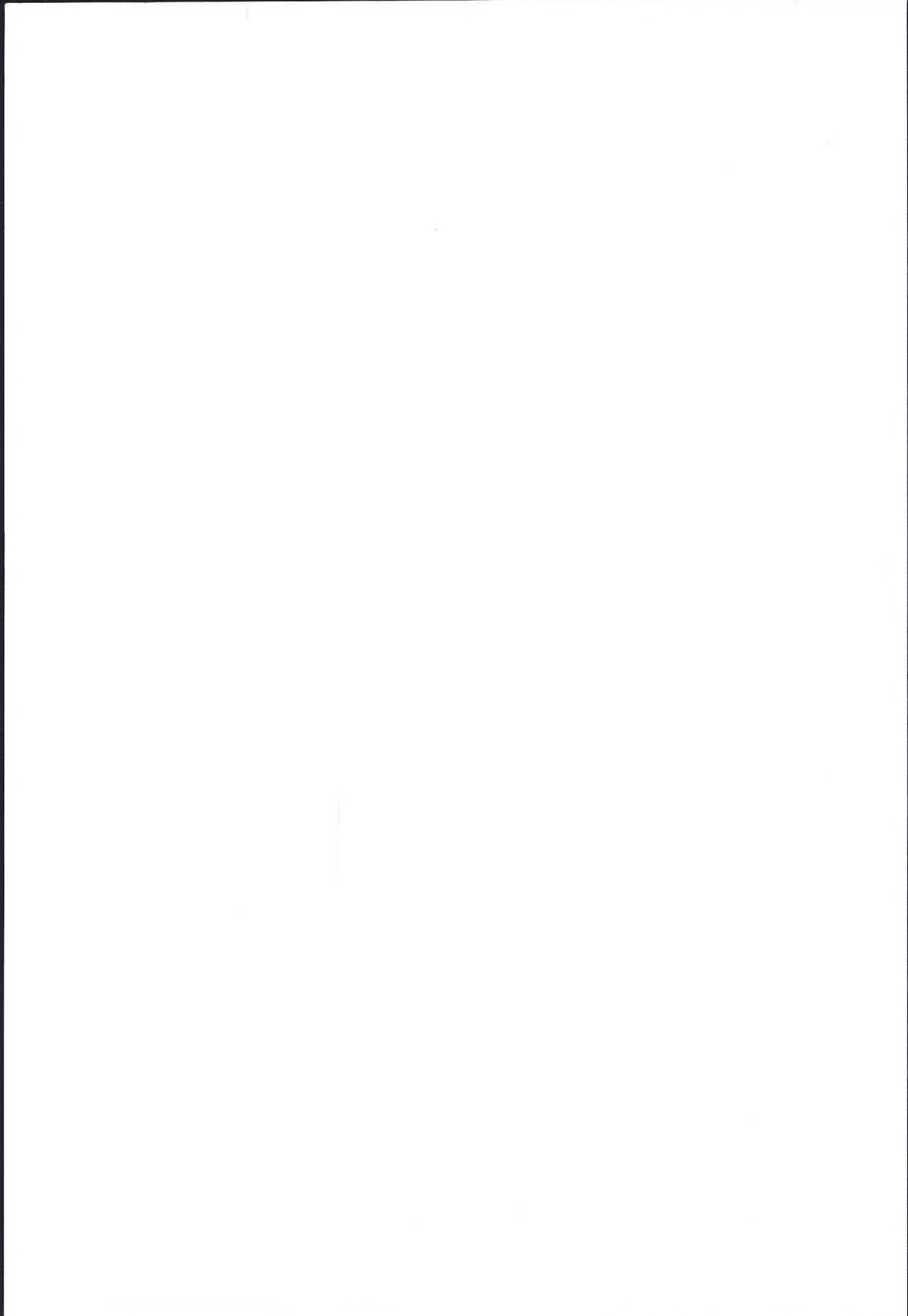
ADVERSE EFFECTS OF DENTAL MATERIALS
Clinical and experimental observations

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Göteborg 1997



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Abstract

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Possible side effects of dental amalgam restorations were investigated in a group of 50 referred patients who related their symptoms to their dental restorations. This group of patients was compared with a matched control group. All patients were subjected to an oral and medical as well as an psychiatric/psychological examination, and the symptoms were related to mercury levels in blood, urine and hair. A psychiatric diagnosis was established in 70% of the patients in the index group versus 14% in the control group. The prevailing symptoms were, anxiety, asthenia and depression. Somatic diseases were more common in the index group (38% versus 6%) and symptoms related to craniomandibular dysfunction were reported by 74% of the patients in the index group versus 24% in the control group, and were diagnosed in 62% and 36%, respectively. Mercury levels in blood, urine and hair were similar in both groups, and far below critical values of mercury intoxication. No correlation was found between mercury levels and the severity of reported symptoms.

The effect of selective replacement of dental amalgam fillings on lichenoid contact lesions (CL) was studied in a group of 142 patients. Ninety-five % of the lesions resolved or improved markedly within an observation period of one year. The corresponding figure for oral lichen planus (OLP) patients were 63%. OLP lesions in sites not in contact with amalgam were not affected. The healing response in patients who received noble gold crowns was superior to treatment with metal-ceramic crowns ($p < 0.05$).

The differential diagnosis between OLP and CL is based on clinical findings, since it is not possible to differentiate by means of histopathological methods. As these two lesions are regarded to have different etiologies, the frequencies of different T cell V-families were examined by immunohistochemistry. It was not possible to discriminate between OLP and CL by this method. No increased expression of any of the investigated V-families was revealed.

The effects of resin components and eluates of root canal sealers were tested in a con-A driven proliferation of both spleen cells and purified T cells which were activated by pulpal cells. The proliferative response, in both assays became suppressed in a concentration-dependent fashion by several of the tested materials. It was noticed that some materials also had a stimulatory effect on spleen cells at low concentrations. These *in vitro* studies show that resin components and extracts from root canal sealers may evoke either immunosuppression or immunostimulation on mitogen-driven proliferation of purified T cells and spleen cells.

The observations made in this thesis support the view that potential adverse effects of dental materials have to be analyzed by different methods which follow formalized and harmonized standards. Thus, information about possible risks should be based on observations from both experimental tests *in vitro* and *in vivo*, and from studies of documented clinical side effects.

key words: mercury, mental disorders, lichenoid contact lesion, hypersensitivity, V β 3, cytotoxicity, immunocompetent cell

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PREFACE

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. Bratel J, Haraldson T, Meding B, Yontchev E, Öhman S-C, Ottosson J-O. Potential side effects of dental amalgam restorations. I. An oral and medical investigation. *Eur J Oral Sci* 1997;**105**: in press.
- II. Bratel J, Haraldson T, Ottosson J-O. Potential side effects of dental amalgam restorations. II. No relation between mercury levels in the body and mental disorders. *Eur J Oral Sci* 1997;**105**: in press.
- III. Bratel J, Hakeberg M, Jontell M. Effect of replacement of dental amalgam on oral lichenoid reactions. *J Dent* 1996;**24**:41-45.
- IV. Bratel J, Dahlgren U, Jontell M. The frequency of different T cell receptor V-families in oral lichen planus and lichenoid contact lesions. An immunohistochemical study. Submitted for publication.
- V. Jontell M, Hanks CT, Bratel J, Bergenholtz G. Effect of unpolymerized resin components on the function of accessory cells derived from the rat incisor pulp. *J Dent Res* 1995;**74**:1162-1167.
- VI. Bratel J, Jontell M, Dahlgren U, Bergenholtz G. Effects of root canal sealers on immunocompetent cells *in vitro* and *in vivo*. Submitted for publication.

ABBREVIATIONS

| | |
|-----------------|--|
| APC | Antigen-presenting cell |
| bis-GMA | Bis glycidyl methacrylate |
| BPA | Bis phenol A |
| CAMP | Camphoroquinone |
| CL | Lichenoid contact lesion |
| CMD | Cranio-mandibular dysfunction |
| Con A | Concanavalin A |
| CPRS | Comprehensive psychopathological rating scale |
| DC | Dendritic cell |
| DSM-IV | Diagnostic and statistical manual of mental disorders, 4th edition |
| DMSO | Dimethyl sulfoxide |
| DTH | Delayed-type hypersensitivity |
| ERCS | Extracted root canal sealer |
| GMA | Glycidyl methacrylate |
| Hg ⁰ | Metallic mercury |
| IBQ | Illness behaviour questionnaire |
| ICD-10 | International classification of diseases, 10th edition |
| IgE | Immunoglobulin E |
| IgG | Immunoglobulin G |
| KSP | Karolinska scales of personality |
| LC | Langerhans cell |
| MACL | Mood adjective checklist |
| MeHg | Methylmercury |
| MHC | Major histocompatibility complex |
| MHLC | Multidimensional health locus of control |
| OLR | Oral lichenoid tissue reaction |
| OLP | Oral lichen planus |
| PFM | Metal-ceramics |
| RCS | Root canal sealer |
| TEGDMA | Tri ethylene glycol dimethacrylate |
| UDMA | Urethane dimethacrylate |

INTRODUCTION

The frequent use of xenobiotics in the industrialized world has raised concern about the possible adverse effects of such materials on living organisms. For most substances scientific tests for possible noxious repercussions have not yet been developed, although a number of regulations have been implemented in an attempt to minimize the risks for the environment in general and for human beings in particular. The usefulness of the measures concerning dental materials is difficult to assess, as possible adverse effects may not invariably be apparent in clinical manifestations. Thus, from both an ethical and a biological perspective, it is imperative that risk assessment strategies, and new technologies, permitting a more selective elimination of deleterious compounds, should be developed.

The frequent use of amalgam restorations in dental care during the last two centuries is one of the main reasons why their possible adverse effects have been scrutinized. Already when it was introduced at the beginning of the 19th century, the use of dental amalgam was subjected to intense criticism, and the American Society of Dental Surgeons later urged their members to abandon the use of amalgam in dental restorations. A more scientific approach was later developed in the management of dental amalgam which led to a specified composition and handling of the material which was accepted by the National Bureau of Standards in the United States later in the 19th century.

The debate about the hazards associated with dental amalgam revived when the German chemist Alfred Stock published an article on the danger of mercury vapour released from dental amalgam (Stock 1926). He held the view that minute doses of mercury were harmful to sensitive individuals. However, the issue lost impetus during World War II.

During the late 1970s an intense debate in the mass media developed in Sweden and in some other countries regarding the possible connection between dental amalgam restorations and a broad spectrum of both somatic and mental symptoms. This created widespread concern and in 1985, the Swedish National Board of Health and Welfare appointed an Expert Committee to investigate the health risks associated with low dose exposure to mercury, in particular in dentistry. This Committee concluded that dental amalgam may have subclinical toxicological effects which justify concentrating resources on research in an effort to develop more technically and biologically suitable dental materials (Socialstyrelsen 1987). However, the committee concluded that there was no available scientific proof that dental amalgam restorations cause overt clinical manifestations of a pathological nature.

In 1992, the Swedish Medical Research Council organized a state of the art conference on health risks associated with dental amalgam. It was stated that "available data do not justify discontinuing the use of any currently available dental restorative dental material or recommending their replacement". Furthermore, it was concluded that no cases of systemic diseases, definitely caused by dental amalgam, have been scientifically documented although allergic reactions to mercury in amalgam fillings may occur (MFR 1992). These conclusions were later confirmed by the Swedish National Board of Health and Welfare following further reviews of recent scientific reports (Socialstyrelsen 1994).

Most of the documented adverse effects of dental materials concern local toxic and allergic reactions in the oral cavity. Reliable estimations of the prevalence of these side effects are lacking, but they are considered to be relatively rare (Hensten-Pettersen 1992, Mjör 1992). In one study the incidence of adverse effects was estimated to be 1:700 for all types of dental materials used (Kallus and Mjör 1991). Even though many dental materials contain substances which are sensitizers including colophony, resin based composites, formaldehyde, eugenol, chromium, cobalt and mercury, it has become evident that allergenic or toxic components do not necessarily elicit allergic reactions.

The intense debate about dental amalgam, both in the scientific community and in society in general, has emphasised the need for clinical studies dedicated to an assessment of the possible long-term effects of dental materials in general, both from toxicological and allergic points of view. Moreover, it is important to improve the quality of experimental assessments, both *in vivo* and *in vitro*, with the aim of studying the effects of unchallenged noxious material on defence mechanisms. The long-term goal for all biocompatibility testing of dental materials should be to inspire the development of new materials for dentistry which do not create risks for human beings or for the environment.

AIMS OF THE THESIS

The purpose of the present thesis was to study various adverse effects that have been associated with dental materials. The specific objectives were to investigate:

- if patients who relate their symptoms to dental amalgam restorations suffer from impaired somatic health (I).
- if patients who relate their symptoms to dental amalgam restorations suffer from impaired mental health (II).
- the healing response of amalgam-induced contact lesions after selective replacement of dental amalgam restorations and to evaluate how different amalgam substitutes facilitate healing (III).
- the possibility of discriminating between amalgam-induced contact lesions and other lichenoid lesions by means of immunohistochemistry (IV).
- the cytotoxic effect of resin composites and root canal sealers upon the function of a mitogen-driven proliferation of spleen cells and T cells activated by pulpally derived accessory cells (V-VI).
- the sensitizing ability of different root canal sealers in a rat model system (VI).

BACKGROUND

The clinical and experimental observations described in this thesis relate to some commonly used dental materials. In this section, (i) known biological features and (ii) possible reactions ascribed to these materials are discussed, (iii) and assessments of dental materials are discussed.

DENTAL MATERIALS

Dental amalgam

Corrosion

It is known that contacts between dental amalgam and precious dental materials cause corrosion currents *in vitro* (Holland 1980) as well *in vivo* (Yontchev *et al.* 1986b). The rate of this current is dependent on different factors such as the potential differences and resistant factors within the amalgam restoration or between the restoration and other dental materials. The anode reaction, often located to the amalgam, releases positive metal ions and electrons. The cathodic reaction in a neutral or slightly alkaline environment causes a reduction of oxygen and water to hydroxide ions ($O_2 + 2H_2O + 4e^- \rightarrow 4 OH^-$), and in a more acid environment the cathodic reaction consists of a reduction of hydrogen ions ($2H^+ + 2e^- \rightarrow H_2$). These corrosion reactions seem to provide a basis for the production of different sulfides leading to the formation of tarnishing substances or precipitation of metal oxides.

Mercury

Pulmonary and gastrointestinal exposure - There is continuous release of metallic mercury (Hg^0) vapour from dental amalgam which is the main contribution to non-occupational exposure. Inhalation of Hg^0 vapour is the main route for uptake and approximately 80% of inhaled Hg^0 vapour is absorbed (Teisinger and Fiserova-Bergerova 1965) and then further distributed to tissues of the body, and accumulated mainly in organs such as kidney and brain (Clarkson *et al.* 1988a, Clarkson *et al.* 1988b, Elinder *et al.* 1988, Hursh *et al.* 1976, Hursh *et al.* 1985). Although the contribution to the body burden of this form of mercury is considered to be small it is not insignificant (Socialstyrelsen 1987). The average daily retention of Hg^0 from dental amalgam has been estimated to be in the range 1,7 to 29 $\mu g/day$ (Berglund 1990, Langworth *et al.* 1988b, Skare and Engqvist 1994, Vimy and Lorscheider 1985, WHO 1991). However, the upper limit value, reported by Vimy *et al.*, has been disputed. This value have been reestimated and it has been suggested that the daily intake should be divided by a factor 16, due to shortcomings of

the methodology described in the paper by Vimy *et al.* (Mackert 1987, Olsson and Bergman 1987).

The retention of the solid form of Hg^0 , which may reach the gastrointestinal tract, have been estimated to be less than 10% (Elinder *et al.* 1988, WHO 1991). Methyl mercury (MeHg) is effectively absorbed (ca. 90%) from the gastrointestinal tract (Elinder *et al.* 1988, WHO 1990), and the main source is provisions. After absorption, more than 90% of the MeHg is transported by blood, and bound to erythrocytes (Elinder *et al.* 1988). MeHg easily crosses different biological barriers and is distributed to all body compartments.

Metabolism - Dissolved Hg^0 in the blood, mainly originating from the vaporous form, oxidizes rapidly in erythrocytes and in other tissues of the body (Hursh *et al.* 1980, Skerfving and Berlin 1985). The reason for this distribution pattern is that Hg^0 vapour instantly penetrate cell membranes, blood-brain barrier and the placenta before an oxidation to mercury ions occur followed by formation and accumulation of different organic mercury compounds in the various extravascular tissues (Skerfving and Berlin 1985). The relative concentration of mercury conjugated compounds is thus related to the oxidation potential of the tissue. The liver, for example, oxidizes Hg^0 effectively through release of the enzyme catalase. Following oxidation, mercury becomes more non-reactive and accumulated in relation to the degree of vascularization. Thus, highly vascularized organs such as the pituitary gland, thyroid gland and kidneys have been reported to accumulate the highest level of mercury (Nylander *et al.* 1989).

Urine and faeces are the main vehicles for excretion. After short term exposure, faecal excretion exceeds the excretion by urine, but after long term exposure urinary excretion dominates, reflecting the slower release of accumulated mercury from the kidneys (Clarkson *et al.* 1988b). Elimination of MeHg occurs mainly through faecal excretion (WHO 1990).

Biological monitoring - Both blood and urinary contents can be used for biological monitoring following mercury exposure. Mercury in the blood is mainly influenced by recent exposure to inorganic mercury and MeHg. As the latter contribute to total blood-mercury concentrations, it conceals mercury released from dental amalgam (Elinder *et al.* 1988, Skerfving and Berlin 1985). However, the plasma concentration of mercury reflects mainly current exposure to inorganic mercury. Mercury concentration in urine relates to previous exposure to inorganic mercury and is probably proportional to the renal burden (Clarkson *et al.* 1988b, Hursh *et al.* 1985). This view is supported by the observation that mercury content in urine is proportional to the number of amalgam surfaces (Langworth *et*

al. 1988a, Molin *et al.* 1990, Olstad *et al.* 1987). Calculations made by Clarkson (Clarkson *et al.* 1988a) based on findings from others (Abraham *et al.* 1984, Patterson *et al.* 1985, Sware *et al.* 1981, Vimy and Lorscheider 1985), show that the urine burden due to mercury release from amalgam fillings may vary between 0.9-5.3 µg/l.

Biological half-time- The biological half-time of inorganic mercury follows a multicompartment model, depending on the duration of exposure and the amount of mercury (Socialstyrelsen 1987). At short-time (20 minutes) exposure with a low dose of Hg⁰, the biological half time was found to be 58 days for the whole body and 64 days for the kidneys (Hursh *et al.* 1976). The half-time for elimination in whole blood after a brief exposure follows a two compartment model with a fast phase of elimination during 2-4 days, accounting for 90 % of the initially deposited mercury, and a slow phase during the following months, accounting for most of the remainder (Barregård *et al.* 1992, Clarkson *et al.* 1988b). In urine, the half-time for elimination of mercury was approximately 40-60 days if a one compartment model is assumed, both after short-time/low dose (Barregård *et al.* 1992) and after long-time/low dose exposures (Skare and Engqvist 1990).

Resin composites

The footing for modern dental resin composites was developed in 1956 by Bowen, when he merged bisphenol A and glycidyl methacrylate, synthesizing bisphenol A-glycidyl methacrylate (bis-GMA) (Bowen 1956). Bis-GMA is the base resin for most of the resin matrix, together with urethane dimethacrylate (UDMA) and triethylene glycol dimethacrylate (TEGDMA). The latter monomer is used as a diluent monomer to attain higher levels of filler particles. Other constituents of composites are inorganic or organic reinforcing filler particles; polymerization initiators (additives that cause the liquid to solidify); polymerization inhibitors (additives that stabilize the liquid until its hardening is desired); silane coupling agents (improve adhesive bonding between resin and the filler particle surface); stabilizers (diminish discoloration of the resin during subsequent ageing)(Bowen and Marjenhoff 1992, Söderholm 1996).

The first resin composites were cured by a chemically activated polymerization process, but today many resins are designed to be cured by visible light. A major concern has been that the polymerization of the resin is far from complete. Ruyter *et al.* found that the degree of conversion of the material was only between 55-73% (Ruyter and Öysaed 1987). Except for the operative advantages with light-curing, it has been questioned if light-curing even gives a lower degree of polymerization, since new sources of failure may be introduced (Öilo 1992).

Biodegradation is a well-known problem of dental composites, causing breakdown and decomposition of the material in saliva and body fluid. This degradation is chemical as well as physical and may be caused by erosion, chewing and bacterial activities (Öilo 1992). Polymers and monomers can be degraded either by oxidation or hydrolysis. It has been shown that from a bis-GMA resin composite, it is mainly unpolymerized bis-GMA or its degradation products that leak from the fillings. The toxicity of the material is reduced by 90% if these leachable products are removed (Rathbun *et al.* 1991). Ferracane and Condon found that 50% of leachable components were eluted within 3 hours and 85 to 100% of leachable components were eluted into water within 24 hours (Ferracane and Condon 1990). Notable is that, formaldehyde, a degradation product of resin composites, was still detectable after 115 days in an *in vitro* experiment (Öysaed *et al.* 1988).

In a recent study, Olea *et al.* suggested that a sealant based on bisphenol-A diglycidylether methacrylate (bis-GMA) increased cell yields and progesterone receptor expression by human breast cancer cells (MCF7). The observed estrogen activity was related to bisphenol-A and bisphenol-A dimethacrylate, which were identified by mass spectrometry (Olea *et al.* 1996). From this study it was concluded that the use of bis-GMA-based resins appears to contribute to human exposure to xenoestrogens.

Regarding the clinical distribution and metabolism of released resin components the information is sparse. However, their potential to be involved in both local and general adverse effects is indisputable as such reactions have been observed although the specific components responsible for these effects still have to be uncovered.

Root canal sealers

Endodontic materials are used in conjugation with filling of instrumented root canals. Most often a combination of a root canal sealer (RCS) and a core is used. The most common core materials are gutta percha, isoprene polymer and metal points. RCS can be divided into 5 categories: zinc-oxide-eugenol sealers; non-eugenol sealers; polymeric resins; rosin-based sealers; and calcium hydroxide sealers (Örstavik 1988).

This diversity of RCS stems from the fact that several different properties have been considered important for the clinical performance of the materials. Thus, zinc oxide-eugenol main property is its sealing ability, preventing ingress of saliva and bacteria to the root canal. Polymeric resins were included to minimize shrinkage of the sealer and calcium hydroxide sealers were introduced as they were suggested to improve the healing potential of apical periodontitis. Paraformaldehyde or other antibacterial agents have been added to obtain an antiseptic effect. Örstavik reviewed the results of several studies examining the

sealing ability against leakage of bacteria and found that zinc-oxide eugenol RCS had good sealing properties; resin based sealers had intermediate sealing properties while resin-based sealers showed the most leakage (Örstavik 1988).

It is known that RCS particles may be found in the surrounding tissues when pulpotomies are performed (Hörsted *et al.* 1982), and they may also initiate local tissue destruction (Rowe 1967, Sjögren *et al.* 1995, Örstavik and Mjör 1992). A systemic distribution of lead and paraformaldehyde, released from N₂ cement, to organs such as, blood, liver, spleen and adrenal gland has been revealed in different animal models (Block *et al.* 1980, Chong and Senzer 1976, Shapiro *et al.* 1975).

Gold-based casting alloys and metal-ceramics (PFM)

Noble/gold-based alloys are used for fabrication of dental inlays and crowns. In Sweden most of the gold-based alloys are of a high noble type, which means that the gold content must be at least 40% (bw), and the total noble metal element content (gold, platinum, palladium, rhodium, ruthenium, iridium, osium) must be at least 60% (bw).

A PFM-restoration is a noble or base metal alloy to which porcelain is bonded via an intermediate oxide layer. In PFM restorations, both palladium and platinum increase casting temperature, strength, and corrosion resistance of the alloy (Anusavice 1996). The major chemical composition of ceramics in PFM restorations, is a vitreous ceramic based on silica (SiO₂) network. Before bonding of the ceramic, the metal alloy is heated in a furnace to burn off remaining impurities and to form a thin oxide layer. This procedure is essential to achieve the bonding of the porcelain to the metal. To obtain various shades and simulate the natural teeth, pigmentsing oxides are added (Mackert 1996).

Even though, noble alloys are regarded to be inert, a measurable release of metallic ions due to electrochemical corrosion has been observed (Lucas and Lemons 1992). Degradation and corrosion have been shown to be dependent on the composition and metallurgic state of the material, as well as host environment and functional aspects of the construction. Sarkar *et al.* reported a dissolution of copper from low gold-based alloys (Sarkar *et al.* 1979).

ADVERSE REACTIONS TO DENTAL MATERIALS

Dental materials have been ascribed various types of adverse effects and not only related to the oral tissues but also to the organism as a whole. Most reactions to dental materials are presumably subclinical which implicate that they are unperceived and do not restrict the living of an individual in a timely manner. Clinical manifestations may be defined as reactions which the individual is aware of and may or may not interfere with daily living. Either reactions may be toxic and/or allergic and affect an individual locally and/or systemically. A toxic reaction is defined as a cell and tissue destruction without activation of any specific defence system *per se*. The toxic reaction is concentration dependent in the sense that higher concentrations cause a more substantial destruction than lower concentrations. An allergic reaction involves natural or specific functions of the immune system where different effector mechanisms cooperate to defend the organism to injury. However, the biologic rationale behind some of the allergic reactions is not easily recognized as they may themselves cause deleterious effects.

Regarding oral tissues, negative reactions affecting defence mechanisms are of particular interest as an impaired system for defence may open avenues for other deleterious matters to the host organism. In order to understand both subclinical and clinical manifestation to dental materials, this part of the background is introduced by a brief description of some cellular components and reaction patterns of the immune system followed by a presentation of toxic and allergic adverse effects of dental materials.

Cellular components of the immune system

Antigen-specific immunity is characterized by an active operation of defence that can specifically recognize and selectively eliminate foreign antigens. The major components of the antigen-specific immune system are lymphocytes and so called antigen-presenting cells (APCs), such as macrophages and dendritic cells.

Lymphocytes

Lymphocytes are of a crucial importance, because they have the ability to discriminate between self/non-self structures. The two main types of T-cells are T helper cells and T cytotoxic cells. T helper cells (CD4+) are important because they do not only recognize foreign antigen but they also play a crucial role in orchestrating the immune response. The cytotoxic T cells (CD8+) are responsible for the elimination of virus infected cells, tumour cells and for graft rejection.

Most lymphocytes are small and inactive as they circulate between the blood and the lymphoid organs. When a foreign antigen enters the body, a proper interactions between

processed antigenic structures, expressed on the cells surface of an APC, and the antigen-specific T cell receptor (TCR) are mandatory for the T cell to be activated. The interactions of these molecules provide the T cell with the first signal. In order to be fully activated, second co-stimulatory signals have to be delivered. By expression of adhesion molecules such as B7, ICAM-1 and LFA-3, the APC can bind to ligands such as CD28, LFA-1 and CD2, expressed by the T cell and deliver deliver the second signal (Steinman and Inaba 1989). During activation, the T cell is also provided with cytokines, a group of biologically active molecules that help to regulate the intensity and/or duration of the immune response by stimulating or inhibiting the activation.

T helper cells are needed in order to activate B cells and cytotoxic T cells. T helper cells occur as, T_H1 or T_H2 depending on their cytokine production profile. T_H1 cells, predominantly produce $IL-2$, $INF-\gamma$, and $TNF-\beta$, and are involved in inflammatory reactions while T_H2 cells produce $IL-4$, $IL-5$, $IL-6$, $IL-9$ and $IL-10$, which are important for the activation of B-cells and induction of an antibody mediated response (Macatonia *et al.* 1993, Mosmann and Coffman 1989).

T cell receptor (TCR) - The TCR consists of two peptide chains. The variability of the TCR is generated from recombinations of V, D and J- gene segments coding for the peptides. The V-region gene fragments are divided into families according to base homologies. Gene fragments belonging to a certain V-region family share 70% base homology. Humans have been estimated to have approximately 48 $V\alpha$ gene segments and 60 $V\beta$ gene segments divided into 18 $V\alpha$ and 25 $V\beta$ families (Klein *et al.* 1987, Thomas *et al.* 1997).

Antigen-presenting cells (APC)

The Major Histocompatibility Complex (MHC) region encodes transplantation molecules, class I and class II molecules. Class I molecules are expressed by virtually all cells of the body except erythrocytes while class II molecules are limited to some cells. Cells expressing one or both of these molecules on their surface have the potential to present antigens to antigen-specific T lymphocytes. Depending on the nature of the antigen, different T cells will be activated. A virus infected cell for example, will produce virus proteins which will be recognized by $CD8+$ T cytotoxic cells together with the class I MHC molecules on the surface of the viral infected cell. The activated cytotoxic T cell will then be able to kill the infected cell, expressing the viral antigen (Roitt *et al.* 1996).

Class II molecules expressing cells have the potential to internalize, process and present extracellular proteins. Following this internal processing of the proteinous antigen, peptides are presented together with class II molecules to $CD4+$ T helper cells which will

be activated given that other necessary signals are provided. Some of these class II molecules expressing cells, such as dendritic cells, are considered as highly effective and have been designated "professional" APCs (Steinman 1991).

Thus, TCR on the T lymphocyte recognises peptide antigens presented on either class I or class II MHC molecules. Some bacterial and viral components, termed superantigens, make exception to this rule as they bind directly, i.e. unprocessed, to the outer surface of the class II molecules and the TCR's from certain V β families (Marrack and Kappler 1990). When traditional microbial antigens are presented by APC:s, less than one in 10,000 of the T-cells may be activated. In contrast, superantigens can stimulate one or several V β families which may arouse as many as 5-10% of all T-cells.

Dendritic cells (DCs)- DCs, comprise a group of cells that display characteristic dendritic morphology, constitutively express high amount of class II molecules, and, as mentioned above, act as potent APCs for both naive and memory T cells. DCs are assembled in a network of APCs which can be found in almost all tissues and organs of the body. The lack of specific DC markers make discrimination to macrophages difficult. DCs are recognized by the combination of the following features: (i) profile of the expression of various cell-surface molecules and/or cytoplasmic markers, (ii) morphological characteristics such as typical dendritic processes, (iii) weak phagocytic activity, (iv) a good endocytic capacity, and (v) potent lymphocyte-stimulating activity *in vitro* (Steinman 1991).

Macrophages- Macrophages play a key role as a member of the phagocytic system and as these cells are recruited to inflamed areas. Inflammatory stimuli cause activation and further differentiation of macrophages. Macrophages derived from various tissue compartments, effect T lymphocytes differently suggesting varying pathophysiological roles in the development and regulation of the immune response (Gong *et al.* 1994).

Reaction patterns of the immune system

In the previous text, some of the cellular components were described to provide a basis for the understanding of immunological reactions such as delayed-type hypersensitivity reaction and oral tolerance, which both are important for the comprehension of how dental materials interfere with the immune system.

Delayed-type hypersensitivity (DTH) reaction

DTH, may occur as contact, tuberculin and granulomatosis reactions. In the first two types the peak is after 48-72 hours, while the granulomatous reaction develops after 21-28 days. All these reactions develop as a result of a repeated contact with the antigen in a sensitized

individual. Experiments with adoptive transfer of sensitized T cells to naive animals have been essential for the understanding of DTH as a T cell mediated reaction. This is further substantiated by the fact that DTH fails, if T cells are depleted prior to the transfer of mononuclear blood cells (Ahlfors and Czerkinsky 1991).

The first step in a DTH reaction is the sensitization phase where an antigen is internalized by MHC-class II expressing APCs, such as Langerhans cells in oral epithelium or dendritic cells of the dental pulp. During the migration to regional lymph nodes, APCs upregulate the synthesis of class II molecules (Puré *et al.* 1990) and are thus preparing for assembling these molecules with processed peptides. Following the entrance to T-dependent area of the lymphoid organs, APCs mature and present the assembly of antigen peptide and class II molecule that will be bound to TCR on the membrane of naive T cells. The activation and proliferation of these cells result in the recirculation of an expanded population of antigen specific memory CD4⁺ T cells. During the effector phase, resident APCs of the exposed tissue have the capacity to capture re-introduced antigen that will then be presented locally to patrolling CD4⁺ memory T lymphocytes. This may cause activation of the memory cells, which triggers recruitment and activation of inflammatory cells including macrophages, the critical effector cells in DTH reactions.

DTH-reaction in the oral mucosa- The Langerhans cells (LC) represent a peripheral outpost and provide the oral mucosa with an immunosurveillant competence (Lombardi *et al.* 1993). LC have the ability to internalize and process antigens, and after migration to the regional lymph nodes, the cells are able to execute their function as accessory cells and present antigens to naive T cells (Steinman 1991). Upon stimulation, T cells clonally expand, enter the blood stream and aquire the ability to traffic all peripheral tissues as memory cells.

When the antigen again challenges the oral mucosa, it is captured and degraded by resident LC. The antigen is then presented to patrolling memory T-lymphocytes, which are activated locally and trigger the effector phase. LC are thought to be the most superior APCs even in the secondary immune response while other class II molecule expressing cells, such as macrophages, keratinocytes and endothelial cells, also acquire this capacity following appropriate stimuli that cause up-regulation of class II and co-stimulatory molecules of these cells.

DTH-reactions in the dental pulp- The normal human pulp is equipped with several immunocompetent cells, such as T lymphocytes and APC cells including macrophages and dendritic cells (Jontell *et al.* 1987). Thus, in principal, DTH in the dental pulp or periapical tissue may not be different from the development of DTH reactions in other tissues.

Although unsupported by experimental data, it is reasonable to assume that, when an antigen reaches the periphery of the pulp from for example a carious lesion, it is endocytosed and processed by DCs. DC migrate through lymph vessels and reach the regional lymphnode where the antigen-presentation to resting T cells takes place. A subsequent clonal expansion and maturation of antigen specific CD4+ T helper cells will be obtained, specific to the foreign antigen. The T cells will then find their way through the blood stream to the site of antigenic challenge in the pulp, and there these cells may orchestrate the local immune response if the antigen is reintroduced (Jontell and Bergenholtz 1992).

Oral tolerance

Tolerance is defined as suppressed activity of certain effector functions of the immune system such as antibody production and activation of inflammatory T cells. Tolerance develops to both self and non-self molecules. Self-reactive T cell clones are destroyed at an early stage in the thymus, which is referred as to central tolerance. Peripheral tolerance may be induced by oral administration of an antigen. Three main mechanisms have been proposed to be involved in maintaining tolerance namely anergy, clonal deletion and suppression (Chen *et al.* 1995)

Oral tolerance was first described by Dakin in 1829 (Mowat 1987) and oral administration of proteins has been claimed to be one of the most effective means to induce tolerance to protein (Tomasi 1980). The T cell dependency of tolerance can be shown by transfer of T cells. Thus, suppressor T cells from spleens transferred from ovalbumin-fed mice to a naive recipient before immunization with this protein suppresses the DTH-reaction (Torii *et al.* 1993). It has also been possible to suppress DTH-reaction in already sensitized animals by feeding them with ovalbumin shortly after immunization (Lamont *et al.* 1988).

Reactions to components of dental materials

Side effects related to dental materials have been considered to be rare and most of the reports are related to amalgam fillings, even when the figures were adjusted in relation to the prevalence of different materials (Kallus and Mjör 1991).

Reactions to dental amalgam

Toxic effects related to mercury - Acute exposure of high concentrations of Hg⁰ vapour may cause pneumonitis (Garnier *et al.* 1981) and long-term exposure provokes critical organs like CNS and the kidneys. Neurological symptoms with tremor have been reported in persons with occupational exposure at urine-Hg level of 50-100 µg/l (Danziger and Possick 1973, Ngim *et al.* 1992). At these urine-Hg levels an increase in urinary proteins

have also been observed (Barregård *et al.* 1988). Other classical symptoms of higher exposure levels are mental changes and gingivitis (Hunter 1975, Shapiro *et al.* 1982).

At lower doses, a non-specific, asthenic-vegetative syndrome called micromercurialism have been suggested to appear (Friberg and Nordberg 1972). The symptoms are more diffuse with fatigue, anorexia, loss of weight, general weakness and disturbances of gastrointestinal functions. It is not clear at which levels micromercurialism emerges, but it has been proposed that an air mercury concentration between 10-100 $\mu\text{g}/\text{m}^3$ (Berlin 1986) causes clinical manifestations.

Lichenoid contact lesion to dental amalgams (CL)- CL is defined as oral mucosal lesions which present with similar clinical characteristics as oral lichen planus, but the degree to which the oral mucosa is involved is different. By definition, CL is limited to areas of frequent contact with dental restorations, mostly dental amalgam, while OLP also involves other regions of the oral mucosa as well (Bolewska *et al.* 1990a, Lind *et al.* 1986). Difficulties in discriminating between CL and OLP have made it hard to obtain prevalence figures for CL. Histopathologically, lichenoid reactions are characterized by hyperkeratosis or atrophy of the epithelium, degeneration of basal epithelial cell-layer which covers a dense band of mononuclear cells, known to consist mainly of T lymphocytes (Dockrell and Greenspan 1979, Sloberg *et al.* 1984, Walker 1976). Various methods have been used in an attempt to separate OLP from CL, with little success (Bolewska *et al.* 1990b, Bolewska and Reibel 1989, Ibbotson *et al.* 1996). •

It is reasonable to assume that CL is the most frequent type of DTH in the oral mucosa. The close association between CL and restorations of dental amalgam lends support to this. This view is also supported by the observation that patients with CL present with an increased frequency (19-52%) of cutaneous DTH to mercury following patch testing (Bolewska *et al.* 1990a, James *et al.* 1987, Nordlind and Liden 1992). The comparable figure in OLP patients is 5% (Bolewska *et al.* 1990a), similar to what has been found in a non-selected population (Holmstrup 1991b). Thus, mercury released by dental amalgam fillings has been suggested to elicit a DTH-reaction in the oral mucosa (Bolewska *et al.* 1990a), although this type of allergic reaction is rare taken into account the frequent use of this restorative material in dentistry. The concentration of released mercury as a sensitizer may not be high enough (Luders 1987) or oral tolerance may develop in individuals who have not previously been sensitized through other routes for ex. the lungs or the skin (Nakayama *et al.* 1983, White and Smith 1984).

The healing of CL lesions in contact with restorations in dental amalgam varies between 87-100% following the replacement (Bolewska *et al.* 1990a, Finne *et al.* 1982, Laine *et al.*

1992, Lind *et al.* 1986, Skoglund and Egelrud 1991). These observations provide additional support for the view that mercury is responsible for the induction of lichenoid reactions from amalgam fillings.

Reactions to resin composites

Resin containing materials may be the single largest sensitizing dental material which may cause contact allergy (Hensten-Pettersen and Jacobsen 1991a, Nathanson and Lockhart 1979). Although, reports on systemic reactions are sparse, local manifestations with the characteristics of a lichenoid reaction have been found in the oral mucosa adjacent to resin based composite restorations (Blomgen *et al.* 1966, Holmstrup 1991a, Lind 1988).

It is also known that resin composites may cause occupational health problems presented as dermatoses on hand and fingers (Kanerva *et al.* 1993, Munksgaard *et al.* 1990). In a questionnaire survey among prosthodontists, 9% responded that they had experienced these problems (Hensten-Pettersen and Jacobsen 1991b). A recent report stated that there is an increasing number of occupationally elicited allergic reactions among dental personal due to resin composites (Edqvist 1995).

Although, pulpal inflammation and necrosis have been associated with resin composites (Stanley *et al.* 1967, Tobias *et al.* 1973). Recent findings support the view that bacterial leakage along these restoration margins, rather than the resin composite *per se*, is responsible for these reactions (Bergenholtz *et al.* 1982, Brännström and Nyborg 1972, Cox *et al.* 1987) (for review see; Bergenholtz 1989, Browne and Tobias 1986). Bacterial leakage between cavity and filling may be attributed to weaknesses in the bonding system and to shrinkage of the material upon setting.

Reactions to root canal sealers

Although side effects to endodontic sealing materials are not frequently seen, there are case-reports on DTH-reactions to eugenol and epoxy-resin containing materials (Barkin *et al.* 1984, El-Sayed *et al.* 1995, Hensten-Pettersen 1984, Hörsted and Söholm 1976). Also cases of a likely formaldehyde-induced anaphylaxis have been reported (Ebner and Kraft 1991). Paraformaldehyde, which is a constituent in some root canal sealers have been shown to cause neurotoxic side effects if the RCS is extruded through the apical foramen (Fanibunda 1984).

Reactions to gold alloys

Contact allergy to gold may be more common than previously anticipated (Björkner *et al.* 1994, Laeijendecker and van Joost 1994). Gold-based alloys have been found to elicit intra-oral contact allergic reaction, and it may be regarded as more potent sensitizer than

less noble so called base-metal (non-precious) alloys (Hensten-Pettersen 1992). When testing 1056 dermatitis patients without any intra-oral manifestations, 15% were found to be gold positive following patch-testing. A positive response to gold was also over-represented in patients having dental gold alloys (Bruze *et al.* 1994).

Oral tolerance and dental materials

As previously mentioned, there are no exact figures on allergic reactions in the oral cavity, but the prevalence figures are expected to be low. This is surprising, in that several dental materials in use are known to be potent sensitizers including eugenol, resins, mercury, gold, palladium and nickel. One reason may be, the phenomenon of oral tolerance. From animal experiments it is known that ovalbumin (OVA) fed rats develop significantly reduced levels of IgG and IgE and DTH responses to OVA (Dahlman *et al.* 1994). Experiments with pigs wearing occlusal splint containing both nickel and chromium or were fed pellets containing these substances did not develop hypersensitivity upon challenge (Vreeburg *et al.* 1984). It has been demonstrated in a retrospective clinical study that children who received orthodontic braces prior to ear piercing showed a reduced frequency DTH to nickel (van Hoogstraten *et al.* 1991). These results supports the possibility that peripheral tolerance may partly explain the low frequency of clinical reactions to dental materials.

IN VITRO AND IN VIVO ASSESSMENTS OF DENTAL MATERIALS

The International Organization for Standardization (ISO) has had a profound impact on the development of biocompatibility standards for dental materials. In Europe, European standards (EN) have been developed by CEN (Committée Européan de Normalisation), and these work in parallel with ISO. For dental products, the most important standard is ISO 10993 (ISO 1992a), which was developed to protect humans against adverse effects of medical devices. This standard comprises 12 different parts. Number 5 (ISO 1992b) contains protocols for cytotoxicity testing. ISO 10993 is supplemented with technical applications in a standard called ISO/TR7405 (ISO/TR 1995).

To gain knowledge as to the noxious potential of a given material, these standards require the use of a multitude of tests including *in vitro* and *in vivo* methodologies. It is then expected that the results are german to the condition under which the material is to be used. Unfortunately, no test method or combination of methods can be said to completely fulfill this task although the relative toxicity can be determined by *in vitro* cell systems employing single target cells. While *in vitro* systems are sensitive and highly able to discriminate one material from another in terms of their potential to cause cell damage and

impaired cell function, *in vivo* tests including implantation tests and so called usage tests provide data as to the temporal feature and the extent of the local cytotoxicity. For example the placement of a restorative material in test cavities prepared in human or animal teeth indicates its irritating potential on the pulp, a common and wide spread methodology for the testing of the biocompatibility of restorative materials. However, both implantation tests and usage tests are restrained by a variety of variables that may conceal any true biological effect of a certain material including the surgical trauma for the placement of the material, the condition of the tissue, the involvement of infection and the occurrence of artefacts upon tissue preparation (Mjör 1980). Maybe the largest drawback of the usage test is its inability to reveal other than local effects. Hence, this test is unable to detect any influences on the organisms as a whole and particularly those manifestations that may appear on a long-term basis.

Also *in vitro* tests are hampered for example by the fact that these tests are carried out on cells which have been taken out of its normal complex environment where otherwise an array of defense mechanisms may be operating. These tests are further unable to account for any secondary effect that may be elicited upon a cytotoxic reaction. Under *in vivo* conditions, cells of the immune system carry out important functions to protect the organism from a large variety of foreign agents. Hence, upon cytotoxic influences both stroma cells and immune cells may be impaired.

Current methodology suggested by the ISO and the European standards do not include testing on the functional capacity of immune cells. Most likely, components from dental materials will be injurious to the oral tissues as well as the local immune system. An impaired immunosurveillant capacity of immune cells, such as Langerhans cells of the oral mucosa and dendritic cells of the dental pulp, following exposure to components released from dental materials, may cause additional adverse effects by inducing either a direct deleterious effect to the tissue or indirectly by providing avenues for the establishment of an infection. Efforts must, therefore, continue to refine and further develop current methodology for biological testing of dental materials.

METHODOLOGICAL CONSIDERATIONS

Oral/medical, and psychiatric/psychological data were collected from patients with the self-diagnose of oral galvanism and the symptoms were related to mercury levels in blood, urine and hair (I-II). In study III, the outcome of selective dental amalgam replacement on lichenoid contact lesions was examined. In order to find an objective method to separate CL from OLP, a standard immunohistochemical technique was used to investigate the distribution of different TCR V-families in the two lesions (IV). In the functional studies (V-VI), the cytotoxic effects of resin composites and root canal sealers was tested on (i) spleen cells and (ii) pulpal APC in a mitogen-driven proliferation assay of T cells.

Since the methodology employed in the present thesis is described in detail in the separate articles, it will only be briefly outlined here.

POTENTIAL SIDE EFFECTS OF DENTAL AMALGAM RESTORATIONS (I-II)

Patients

A group of 50 patients (29 women and 21 men; mean age = 52.2 yr; range = 20-80 yr) was referred to the Department of Oral diagnosis, Faculty of Odontology, Göteborg University for self-related complaints of oral galvanism or mercury poisoning. A group of 50 healthy controls (mean age = 51.7 yr; range = 20-80 yr), randomly selected from the Public Dental Service was matched to the index group by age, sex and postal zip code.

Methods

Both groups were subjected to a general oral examination including registration of number of teeth and tooth surfaces with different types of dental restorations. The study also comprised a stomatognathic examination, an examination of the surface corrosion state of dental restorations, patch testing, test for mercury in whole blood, plasma, urine and hair, and a clinical chemistry examination.

The *stomatognathic examination* was based on an anamnestic and clinical dysfunction index by Helkimo (Helkimo 1974). Background variables were analysed by using a self-administrated questionnaire developed by Carlsson *et al.* (Carlsson *et al.* 1982).

An inventory, the *Check List of Symptoms (CLS)* was used collecting 59 symptoms related to four regions; the mouth, the teeth, the head/neck and the rest of the body.

The *surface corrosion state* of dental fillings examination used in this study was the same previously reported (Yontchev *et al.* 1986b).

Patch testing was performed, using the "dental-screening" protocol consisting of 24 substances (Chemotechnique diagnostics, Malmö, Sweden).

The *clinical chemistry examination* was performed at the department of Clinical Chemistry, Sahlgrenska University Hospital, Göteborg using standard procedures. Whole blood samples were analysed for hemoglobin, erythrocytes, mean erythrocyte volume, mean erythrocyte hemoglobin content, mean erythrocyte hemoglobin concentration, folates, leukocytes including neutrophils, basophils, eosinophils, monocytes and lymphocytes, large unstained cells, trombocytes and erythrocyte sedimentation rate. Analyses were made in plasma or serum for iron, total iron binding capacity, ferritin, cobalamins, folates, thyretotropin, antimicrobial antibodies, gastrin, pepsinogen and zinc.

Mercury levels were determined in whole blood, plasma and urine by a cold vapour atom absorption method, at the National Institute for Working Life, Solna, Sweden (Vesterberg 1991). The values of red blood cells was calculated as the difference between whole blood and plasma. Samples of hair were analysed by the Swedish Environmental Research Institute, Göteborg, Sweden.

Psychiatric examination

Mental disorders were classified after consensus had been reached between two psychiatrists who followed the International Classification of Diseases 9th ed. (ICD-9) and the Diagnostic and Statistical Manual of Mental Disorders, (3 rd revised ed. DSM-III-R, American Psychiatric Association). The diagnoses were later adopted to ICD-10 and DSM-IV.

Psychological examination

In addition to a psychiatric interview the patients were assessed with several rating scales and questionnaires.

CPRS is based on a clinical interview and includes 40 reported and 27 observed psychopathological items, rated from 0 to 3. The 67 items are divided up into 13 groups (Åsberg *et al.* 1978).

The *Zung scale* is a self rating scale of depression. It consists of 20 items, scored 1-4 (Zung 1965).

The *KSP* consists of 135 items pertaining to 15 personality traits, such as disposition to various forms of anxiety, aggressiveness and impulsiveness. The items are scored from 1-4 (Schalling *et al.* 1987).

The *MACL* consists of 71 mood-describing adjectives forming 6 dimensions of emotions obtained with factor analyses: pleasantness, social interaction motive, social orientation, activation, tension and control (Sjöberg *et al.* 1979). The assessment is regarded to reflect the habitual emotional state.

The *MHLC* assesses control of health and confidence in health care and doctors. It consists of 18 questions divided into 3 Health Locus of Control dimensions: internal, powerful others and chance. The questions are scored from 1-6 (Wallston *et al.* 1978).

The *IBQ* consists of seven dimensions of non-adaptive illness behaviour derived from factor analysis: hypochondriasis; disease conviction; disease apprehension; emotion inhibition; emotion disturbance; denial and irritability. Each item is scored 0 or 1, where 1 reflects disturbance. We used a shortened version of 30 items, associated with abnormal disease behaviour (Pilowsky and Spence 1978).

The *Life Event Scale* (Paykel and Myers 1969) records 26 life events to which pregnancy was added. The events are classified in five categories: employment, family, marital, health and legal. We stated the frequency of individuals reporting at least one event in each category within 12 months prior to the onset of symptoms. The controls were asked for events which occurred within one year prior to the interview.

EFFECT OF REPLACEMENT OF DENTAL AMALGAM RESTORATIONS ON ORAL LICHENOID TISSUE REACTIONS (III)

Patients

Patients referred to the Department of Oral diagnosis, Faculty of Odontology, Göteborg University, for OLR were included in this study. OLR irrespective of size were clinically characterised as white lesions with a reticular to plaque-like pattern which displayed a diffuse transition to the surrounding normal oral mucosa. In addition, lesions were included which presented with various degree of atrophic and erosive tissue reactions. The patients were separated into two groups with respect to the involvement of the oral mucosa. CL were defined as lichenoid tissue reactions in contact with restorations of dental amalgam and no OLR were identified in any other areas of the oral mucosa (CL; n = 142; mean age = 58 yr; range = 33-89 yr; 80% women). If patients who presented with

OLR in contact with restorations of dental amalgam and displayed lesions in the gingival tissue, palatal tissue or in the floor of the mouth these lesions were designated oral lichen planus (OLP; n = 19; mean age = 54 yr; range = 36-71 yr; 74% women).

Methods

The majority of the lesions were subjected to histopathological examination and showed microscopically features of a lichenoid tissue reaction i.e. hyperortho-hyperparakeratosis, basal liquefaction and a well demarcated subepithelial infiltrate of lymphocytes.

After examination, restorations of dental amalgam which were in contact with OLR in both OLP and CL groups were replaced by the dentist who had referred the patient. The outcome of the treatment was evaluated after 6-12 months following a clinical score system: no healing = persistent lesions or insignificant improvement of the condition; partial healing = a marked improvement but a discrete white reticular or plaque-like reaction pattern remained; total healing = normal oral mucosa. The type of replacement material used in a randomly selected subpopulation with CL (n=110) was identified and recorded. Medical history and symptoms were recorded in all patients.

THE FREQUENCY OF DIFFERENT T CELL RECEPTOR V-FAMILIES IN ORAL LICHEN PLANUS AND LICHENOID CONTACT LESIONS (IV)

Patients

Biopsies were obtained from 10 patients with OLP (7 woman and 3 men; mean age = 53 yr; range = 34-62 yr) and 10 patients with CL (6 woman and 4 men; mean age = 58 yr; range = 38-71 yr). The same definition was used for both CL and OLP as in the previous study III.

Methods

Cryosections were fixed in a cold acetone/water solution (50:50 bv) for 30 s and subsequently in 100% acetone for 5 min. Following washing in Tris buffer solution (TBS 0.05M, pH 7.6), the sections were exposed to H₂O₂ (0.3%) in TBS for 5 min to deplete endogenous peroxidase activity and then blocked with normal rabbit serum (1:50 in TBS; Dako A/S, Glostrup, Denmark) or dissolved milk powder (5%) for 30 min. Mouse MoAbs directed against the different T cell receptor a and b variable region regions were used as primary antibodies. The incubation was carried out overnight in a humid chamber at 4°C. The following day the slides were further incubated for 30 min with biotinylated F(ab')₂ fragments of rabbit anti-mouse immunoglobulin. Avidin and biotinylated horseradish peroxidase solution (ABCComplex/HRP; Dako) was applied for 30 min and the

peroxidase activity was developed by incubation for 10 min in 3-amino-9-ethyl-carboxyl (10 mg; Sigma Chemical Company, St Louis, USA) dissolved in dimethyl-sulphoxide (6 ml dissolved in 50 ml sodium acetate buffer, 0.02M, pH 5.5) containing 4 μ lH₂O₂ (30%). The sections were counterstained with Mayer's haematoxylin. Negative control staining was made by omission of the primary antibody.

Antibodies

Mouse monoclonal anti-human TCR V-family MoAbs antibodies with the following specificities were used: V β 5a (IgG1, clone 1c1), V α 2 (IgG2a, clone F1) (T Cell Sciences, Cambridge, MA), V β 3 (IgG1, clone LE 89; Serotec Ltd, Oxford, UK), V β 3.1 (IgG1, clone 8F10; Serotec Ltd, Oxford, UK) and V β 3 (IgM; clone CH92; Immunotech, Coulter Co, Marseille, France). All primary antibodies were diluted 1:100 in TBS containing 4% bovine serum albumin. Biotinylated F(ab')² fragments of rabbit anti-mouse immunoglobulin were used as secondary antibodies (1:400; Dako A/S, Glostrup, Denmark).

Measurement of cell distribution in cryosections

Three high-power fields (x400) were selected in areas with strong infiltrates of T lymphocytes and the number of positively stained cells was enumerated by using a 10-mm x 10-mm ocular grid and a x40 objective. In each area, a minimum of 150 cells was counted. Results were expressed as the mean count of 3 high-power fields for each section.

EFFECTS OF RESIN COMPONENTS AND ROOT CANAL SEALERS ON IMMUNOCOMPETENT CELLS (V, VI)

Animals

In each experiment, tissues were obtained from 8-12 week old, male or female Lewis rats (150g). In the experiments studying DTH, performed at the Royal Dental College, Copenhagen, Denmark, 8-10 week old SPF Wistar rats were used (paper VI). The rats were sacrificed with CO₂ inhalation. An animal use protocol was reviewed and approved by Animal Ethic Committees at Göteborg University and Royal Dental College.

Test materials

Various resin components (Paper V; Table 1) were dissolved in 100% DMSO (silylation grade; Pierce, Rockford, IL, USA), as previously described (Hanks *et al.* 1991). Each stock concentration was diluted x1000 in medium which means that the final concentration of DMSO for each test substance was 0.1%. This DMSO concentration gave no statistical variation from controls with any metabolic parameter tested (Hanks *et al.* 1991). The stock solutions were used to make serial non-turbid dilutions, which were added to the cells utilized in the *in vitro* assay.

Table 1. Components of resins, abbreviations, and lot numbers.

| Material | Abbreviation | Lot No | Content |
|---|--------------|-----------|------------------|
| Bis Glycidyl Ether of Bis Phenol A | BGE-BPA | 825 | Precursor |
| Bis Glycidyl Methacrylate | bis-GMA | 334-27 | Major oligomer |
| Bis Phenol A | BPA | 215 | Precursor |
| Camphoroquinone | CAMP | 3111AJ | Photo-absorber |
| Ethoxylated Bis Phenol A Dimethacrylate | E-BPA | PB-1774 | Precursor |
| Glycidyl Methacrylate | GMA | 274-16 | Precursor |
| 1,6 Hexane diol Dimethacrylate | HDDM | 887-315-2 | Monomer |
| N,N Dihydroxyethyl-p-toluidine | DHEpT | 359-16-9 | Accelerator |
| Tri Ethylene Glycol Dimethacrylate | TEGDMA | 334-2 | Diluent oligomer |
| Urethane Dimethacrylate | UDMA | 326-28 | Major oligomer |

Eluates from four different root canal sealers (ERCS; Paper VI) were used in the study; AH 26, Grossman's sealer, Endométhasone and Apexit (Table 2). Materials to be tested were extracted from freshly mixed or solid mixed samples (ISO 1992b).

Preparation of pulpal cells

The teeth were split open and the pulps were dissected (Linde 1972) and thoroughly washed in Dulbecco's modified Eagle's medium (DMEM) containing 2 mmol/L glutamine, 10% heat-inactivated fetal calf serum, and gentamycin (20mg/L). Pieces of pulpal tissue (0.1 mm³) were cut and incubated in DMEM containing 0.5 mg/ml collagenase (Type V; Sigma Chemical Company, St Louis, USA) for 30 min at room temperature. The enzymatic treatment was repeated twice on the residual, non-disintegrated tissue. A final collagenase treatment was carried out in 37⁰ C for 30 min. The obtained cell suspensions were pooled and incubated at 37⁰ C with 0,0025 % DNAase (Type I; Sigma Chemical Company, St Louis, USA) for 3 min to prevent cell clumping. Erythrocytes and dead cells were removed by centrifugation in a density gradient (Ficoll-Paque; Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The pulpal cells were washed twice in DMEM and counted.

Table 2. Content of the used Root Canal Sealers

| Material | Manufacturer/ Batch No | Powder: Hartz: Powder: Liquid: Powder: Liquid: Base: Activator: | Content | % |
|----------------------|--|--|---|---|
| AH26: | De Trey Denitsply, Konstanz, Germany, B:930517 | Powder: | Silver powder Wismutoxid Methenamin Titan(IV)-oxid | 10 60 25 5 |
| Grossman's sealer: | Pharmacy, Glasgow Royal Infirmary, UK, 308003 | Hartz: Powder: | Epoxybisphenolhartz Zinc oxide Stybelline resin Bismuth subcarbonate Barium sulphate Anhydrous sodiumborate | 42 27 15 15 1 |
| Endométhasone ivory: | Spécialités Septodont, Saint-Maur, France B 2038 | Liquid: Powder: | Eugenol Dexametazone Hydrocortisone acetate Thymol iodide Paraformaldehyde Radiopaque exipient q.s. ad | 0.01 1 25 2.2 100 |
| Apexit: | Vivadent Schaan, Liechtenstein, 562526 | Liquid: Base: Activator: | Eugenol Calcium hydroxide Hydrogenized colophony Highly dispersed silicon dioxide Calcium oxide Tricalciumphosphat Zinc stearate Trimethylhexandioldiasalicylat Bismuth carbonate basic Highly dispersed silicon dioxide 1,3 Butandioldiasalicylat Hydrogenized colophony Tricalciumphosphat Zinc stearate | 32 32 8 6 4 2 25 18 15 11 5 5 1 |

Preparation of T-lymphocytes

Dissected lymph nodes (cervical in paper V and mesenteric in paper VI) were gently disrupted with a forceps. Cells were suspended in DMEM and incubated with a mouse monoclonal anti-rat class II molecule antibody (Ox6; Sera-lab Ltd, Sussex, England) in a dilution of 1:100 over night at +4°C. Dynabeads M-280 (Dynal A.S., Oslo, Norway) coated with goat anti-mouse-IgG were added and incubated for 30 min on ice in a cell:bead ratio of 1:20. Class II molecule expressing cells, rosetted with beads, were separated from the remaining non-rosetted cells by a magnet.

Incubation and harvesting procedure

The different cell suspensions were incubated in medium containing concanavalin A (con A; 5 µg/mL, Pharmacia Fine Chemicals, Uppsala, Sweden) for 48 hrs in a CO₂-incubator. ³H-thymidine (5 µCi/mL, Amersham, UK) was added to each well for a continued incubation over another 24 hrs. The cells were harvested by a Skatron harvester (Flow Laboratories, Oslo, Norway), washed and the radioactivity was counted by liquid scintillation.

Assay on spleen cells

To determine concentration intervals at which extracts or eluates of the different test materials would be suitable for assay, tests were run on concanavalin A-induced proliferation of spleen cells. Following dissection, the splenic capsule was disrupted and the cells were released with forceps. After washing twice in DMEM, the cells were placed on a Ficoll-Paque density gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) to remove non-viable cells and erythrocytes. The splenic cells were then washed, counted and transferred to 96-well round bottomed polystyrene tissue culture dishes (Costar, Costar Corp. Cambridge, MA, USA) at 2.5 x 10⁵ cells/ well in 200 µL of DMEM. The cells were incubated in medium different concentration of the resin composite components and ERCS. The incubation and proliferation assay were performed as described above.

Assay on pulpal cells and T-lymphocytes (V, VI)

Purified T-lymphocytes (2x10⁵ cells well) were incubated with pulpal cells (10⁴ cells per well) in the presence of con A with each of the predetermined concentrations of 5 selected resin composite components (V).

The individual susceptibility of accessory pulpal cells and T-lymphocytes to some of the resin composite components was observed in separate experiments (V) . Prior to assay, suspensions of each of these cells were pretreated with GMA (50 µmol/L), bis-GMA (25 µmol/L), UDMA (25 µmol/L) and CAMP (10 µmol/L) for 4 hours in 37°C. The concentrations selected were within the concentration slopes predetermined in the spleen

cells assays described above. Following pretreatment the cell suspensions were repeatedly washed in DMEM to remove residues of the resin.

Suspensions of the pulpal cells were pretreated with various concentration of the different ERCS for 4 hours in 37°C (VI). Following pretreatment the cell suspensions were repeatedly washed in DMEM to remove residues of the ERCS. T-lymphocytes ($2,5 \times 10^5$ cells per well) were added and the incubation and harvesting procedures were performed as described above (10^4 cells per well).

Test for DTH (VI)

Polyethylene tubes with a length of 10 mm and an external diameter of 2 mm (NIOM, Scandinavian Institute of Dental materials, Haslum, Norway), were filled with each of the four different RCS. Mixing of the materials were performed according to the manufacturers' instruction. Prior to implantation, the tubes with the mixture were set during 24 h, 37°C and 97 % humidity. Following rinse in 70% ethanol, the tubes were placed in a cartridge and mounted in an injection device (Kallus and Eklund 1983).

The Wistar rats were administered 0.1 ml/100g body weight of Immobulon^R (Pharmacia & Upjohn, Sweden). The backs were shaved and four tubes containing the same root canal sealer were implanted subcutaneously by injection in each of three animals. Four animals served as controls and received empty tubes. Two weeks after implantation, the animals were, during ether anaesthesia, given an injection into the left ear with 20 µl of relevant ERCS in non-toxic concentrations (AH 26 1:100, Apexit 1:10, Grossman's sealer 1:50, Endométhasone 1:100). In the right ear of each animal, 20 µl of PBS was given subcutaneously as a control. The ear thickness was measured using an Oditest (Kroepelin, Schlüchtern, Germany), before the injection and after 24 h. The animals were sacrificed and the spleens were removed for a spleen cell assay according to the protocol described above. A total of $2,5 \times 10^5$ spleen cells in a final volume of 200 µl were dispensed in each individual well of tissue culture dishes (Costar, USA). For activation, 10 µl of the appropriate, non-toxic concentration of the relevant ERCS was used.

STATISTICAL ANALYSES

All data obtained were computerised and the SAS (Statistical Analyses System) was used for calculations. Student's paired t-test was used for tests of differences between the groups for parametric variables, and the Wilcoxon test was used for non-parametric analyses. In the patch testing examination the Chi-square test was used. Pearson's product-moment correlation coefficient (r) was calculated (study I-II).

Chi-square test, Wilcoxon test and Fisher's exact test were used to test statistical differences in study III, and Mann-Whitney's test in study IV.

Student's t-test was used to test statistical differences in study V-VI.

The descriptive statistics were presented as mean, standard deviation (SD), and standard error of mean (SEM). The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

These results are discussed in the following four sections:

- A. POSSIBLE REACTIONS TO MERCURY FROM DENTAL AMALGAM RESTORATIONS (I-II)
 - B. EFFECT OF REPLACEMENT OF DENTAL AMALGAM RESTORATIONS ON ORAL LICHENOID TISSUE REACTIONS (III)
 - C. IMMUNOHISTOCHEMISTRY IN DIFFERENTIAL DIAGNOSIS OF ORAL LICHENOID TISSUE REACTIONS (IV)
 - D. EFFECTS OF RESIN COMPONENTS AND ROOT CANAL SEALERS ON IMMUNOCOMPETENT CELLS (V, VI)
-

A. POSSIBLE REACTIONS TO MERCURY FROM DENTAL AMALGAM RESTORATIONS (I-II)

A group of 50 consecutive patients, referred for self-reported complaints which they related to amalgam fillings, was compared with a control group of individuals.

Somatic observations

Oral findings - The oral health status which included, periodontal conditions, oral medicine status and number of teeth ($24.5 \pm 6.1SD$ versus $23.8 \pm 6.6SD$) revealed no major differences between the 2 groups, except for some minor differences. There were for ex. significantly more root canal treatments and temporary restorations ($p < 0.05$) in the index group. The higher frequency of root canal therapy in the index group may indicate a greater consumption of dental care due to a more advanced caries situation. From the present study it is not possible to rule out the possibility that medication, as a part of an impaired health situation in the past, had resulted in the development of caries.

The index group also showed more temporary crowns and fillings, which indicated an ongoing dental treatment. Some of these patients may already have started to replace their dental amalgam restorations. By requesting more treatment, the treatment *per se* can result in more root-canal therapy as a complication. It has also been suggested that psychotropic

drugs may be one of the explanations of the higher frequency of dental caries by reducing the saliva flow. However, this hypothesis is opposed by the finding that the salivary variables were within the reference values and did not differ significantly between the two groups. Besides, they were found to be in agreement with previously reported data (Hampf *et al.* 1987, Yontchev and Emilson 1986).

In the examination of the temporomandibular joint system (TMJ) the index cases showed significantly more positive findings than the control group. When, cranio-mandibular disorders (CMD) were assessed using the mandibular dysfunction index, the index group reported "mild" to "severe" symptoms in 74% compared to 24% in the control group ($p < 0.001$). Clinical characteristics of moderate and severe CMD revealed by the clinical dysfunction index, were found to be 62% in the index group versus 36% among the controls ($p < 0.001$). The most predominant symptoms in the index group obtained from the questionnaire related to TMJ were headache, dizziness, globus in the throat, clicking, feeling fatigue in the jaws and clenching of teeth. These findings are well in agreement with previous studies (Jontell *et al.* 1985, Yontchev *et al.* 1986a). The frequent occurrence of CMD is an important observation as it may be successfully treated (Haraldson 1985).

Measuring surface corrosion state of dental fillings and constructions was included in this study because of the concern that high currents could impair health status, due to an increased mercury release. The mean values of the electrode potentials of amalgam fillings were -190.7 mV (SD=132.0) for the index group and -220.4 mV (SD=44.1) for the controls. The two most negative values were measured on a control individual (-592 mV) and an index patient without oral symptoms (-570 mV). The controls had significantly more negative electrode potentials than the index group ($p < 0.01$). When currents were calculated, using measurements with and/or without intermittent contact between fillings and constructions, there was no difference between the 2 groups. Thus, it was unlikely that corrosion products were responsible for the metallic or other diverging taste sensations in the index group. The electrode potential was the same as the previously reported (Yontchev *et al.* 1986b)

General findings - Symptoms from the head/neck and the rest of the body recorded by the CLS were more frequently reported among the index patients, and similar observations have been made in a previous study by Jontell *et al.* (Jontell *et al.* 1985). The index group patients reported significantly more somatic diseases (38% versus 6%; $p < 0.001$), such as cardiovascular disease and rheumatoid arthritis. However, somatic illness was not reflected in the clinical chemistry analyses which were on the whole similar for both groups. The laboratory findings displayed 1 patient with hemochromatosis in the index group, and since this disease has the same typical symptoms as mercury poisoning (Ritter

and Olsson 1988), i.e., tremor and asthenic complaints, a clinical chemistry analysis may be of value in patients presenting with these symptoms. The importance of clinical chemistry investigation is further supported by the finding of generally lower levels of serum pepsinogen in the index group, which may indicate a higher prevalence of atrophic gastritis. It should be emphasized that the number of positive findings are low, but this type of examination may be a valuable contribution to an accurate examination of this patient group.

Psychiatric and psychological observations

A clinical psychiatric diagnosis was established in 70% of the index patients versus 14% of the controls ($p < 0.001$) using both the ICD-10 and the DSM-IV system. According to ICD-10 the dominating diagnoses were "neurotic", "stress-related", "somatoform disorders" and "mood-disorders" and according to DSM-IV the prevailing diagnoses were "anxiety disorder" (38% versus 8%), "somatoform disorder" and "mood disorder". These findings were well in accordance with findings by others (59-73%) using the same diagnostic criteria listed in DSM-III-R (the diagnoses were later adopted to DSM-IV) (Hampf *et al.* 1987, Herrström and Högstedt 1993).

In the CPRS, the index patients showed significantly higher scores on reported "depression", "anxiety", "asthenia" and "vital symptoms" (appetite, sleep, sexuality) and "senso-motor symptoms" (impairment of sensory or motor functions) than the controls. The "global rating" indicated a moderate severity of illness among the index patients. In the CPRS-inventory, the index cases reported significantly more symptoms than the controls while there were no differences between the observed clinical findings. This was a consistent observation and may be interpreted as a result of a systematic bias. However, most likely the patients perceived their symptoms as more serious compared to what may be discerned by the examiners.

In the IBQ-test the index patients scored significantly higher in "hypochondriasis", "disease conviction", and "emotion disturbance" but significantly lower in "disease apprehension" clearly indicating that these patients were more inclined to non-adaptive reactions to bodily discomfort, which agrees with findings by others (Jontell *et al.* 1985). However, our findings were considerably lower than those found by Persson *et al.* (Persson and Svensson 1982). The time aspect could be considered the case, as conviction of mercury poisoning from dental amalgam in relation illness, may have become more widespread and thereby affecting several more groups of people during the middle and end of the 80s compared to the beginning of the decade (Molin and Nilsson 1990). A "disease behaviour" may not have been developed to the same extent by the present group compared to the group examined by Persson *et al.*

Patients in the index group displayed a polysymptomatology which was referred to mercury, even though many of the symptoms did not relate to the classical signs of mercury poisoning. However, it is known that somatic symptoms may be a sign of depression and mental distress if the symptoms are multiple and they may be vague and refer to any body system and function (Lipowski 1990). In our study, the index patients did not show more diagnoses of depression than the controls, but the index group expressed more depressive symptoms not only supported by the Zung depression scale but also by the MACL where the index patients showed significantly lower degree of harmony than the controls.

Mercury burden

The number of amalgam surfaces were correlated to inorganic mercury in plasma and urine, and no significant differences between index and control group were found (28.2 ± 18.9 SD versus 29.0 ± 15.0 SD). Other sources of exposure to inorganic mercury were occupational and fish consumption derived from provisions. The mercury levels were low and showed virtually identical values in both blood and urine (blood: 14.2 ± 11.7 SD versus 15.5 ± 10.0 SD nmol/l; urine: 25 ± 18.3 SD versus 21.9 ± 24.0 SD nmol/l). These values were similar to what have been reported elsewhere among patients with the same kind of complaints (Herrström and Högstedt 1993, Jontell *et al.* 1985). A positive correlation between the number of amalgam surfaces and mercury levels in plasma and urine independent of group (both $r=0.43$), is also in agreement with observations made by others (Langworth *et al.* 1988a, Olstad *et al.* 1987), showing that not only urine but also plasma reflects mercury exposure from amalgam fillings (Barregård 1993). Analyzes of hair samples showed no positive correlation to the number of amalgam fillings, thus hair does not seem to be suitable for monitoring the accumulation of inorganic mercury (Socialstyrelsen 1994, WHO 1991).

Risk assessment of mercury from dental amalgam

Higher levels than 100 nmol/l of mercury in urine indicate an occupational exposure (Skerfving and Berlin 1985). Both groups in our study showed the same mercury levels as reported for non-occupationally exposed individuals (Barregård 1993), and thus far below the level of 250 nmol/l at which discrete renal or CNS symptoms may appear (Barregård *et al.* 1988, Langworth *et al.* 1992, Skerfving and Berlin 1985). In an American study it was shown that mercury exposed dentists with a mean urinary mercury level of 180 nmol/l compared to non-exposed dentists, with a mean mercury level of 25 nmol/l urine, showed an impaired behavioural function and a lower mood score (Echeverria *et al.* 1995). Dentists with a mean blood mercury level of as low as 50 nmol/l have been observed to perform worse in neurobehavioural tests compared to controls

(Ngim *et al.* 1992). However, in a group of chloralkali workers, with the same levels of mercury level, were not observed to deviate from controls in neurobehavioral tests, but slight personality changes were revealed (Langworth *et al.* 1992).

In the present study, several factors argues against mercury as an explanation to the symptoms reported by the index patients. First, the fact that the levels of mercury were similar in the index group and controls and far below the level at which symptoms have been observed (Barregård *et al.* 1988, Skerfving and Berlin 1985, WHO 1991). Second, no significant correlation was found between the mercury levels in tissue fluids and the severity of the oral and mental symptoms. The same conclusion was drawn from observations in a Swedish epidemiological study on women where no correlation between number of amalgam fillings and symptoms was found (Ahlqwist *et al.* 1988). However, in single cases with elevated urinary mercury levels it cannot be excluded that amalgam fillings may contribute to illness (Langworth and Strömberg 1996).

Somatization of mental distress

It is also known that somatic symptoms may be generated by mental distress in a somatization process (Carlsson and Jern 1982). This is not surprising as people in general prefer a somatic explanation to illness, a conception supported by the focus to somatic diseases in health care systems developed in western-oriented societies. Other explanations have a low acceptance and when a somatic diagnosis is not provided by the medical profession, the affected patient seeks a somatic disease to justify illness to herself and the surroundings. These circumstances may have created a distrust of the public health care system. This hypothesis is supported by the MHCL in the present study, where the index patients scored significantly lower in the "internal" and "powerful others".

Release of mercury from amalgam fillings, as a potential cause of various diseases, has received much attention by the massmedia. This has often been mentioned as a contributing factor to the transfer of focus from mental distress to somatic diseases. However, the role of the massmedia has never been fully investigated (Hampf *et al.* 1987, Herrström and Högstedt 1993, Jontell *et al.* 1985, Molin 1992).

In conclusion, no support was found that mercury released from dental amalgam contributes to mental disorders or somatic diseases among the investigated patients.

B . EFFECT OF REPLACEMENT OF DENTAL AMALGAM RESTORATIONS ON ORAL LICHENOID TISSUE REACTIONS (III)

Healing response to selective replacement of dental amalgam

Lichenoid contact reactions (CL) is exclusively a clinical diagnosis, based on the local contact between the lesion and a dental material. In this study, a group of patients with CL in contact to amalgam restorations showed a considerable clinical improvement in 95% of the patients after selective replacement of the restorations and a total relieve of symptoms was obtained. Similar response was not observed following selective replacement of amalgam fillings in contact with oral lichen planus (OLP) lesions. In the OLP group only 63% of the patients with amalgam-associated erosive and atrophic lesions showed an improvement following selective replacement. OLP lesions in sites other than those in contact with dental amalgam restorations were not affected and 53% of the patients still reported symptoms after replacement compared to 79% prior to treatment. These observations support previous results obtained following replacement of amalgam restorations in patients with lichenoid tissue reactions (Bolewska *et al.* 1990a, Ibbottson *et al.* 1996, Skoglund and Egelrud 1991).

Patch testing

In studies were careful discrimination between CL and OLP were performed (Bolewska *et al.* 1990a), close to 50% of CL patients were found to be positive to mercury by patch testing on the skin. In patients with OLP, positive patch testing to mercury was not found to be different from individuals presenting with lichenoid reactions. The high frequency of negative tests in CL patients, i.e. approximately 50%, does not fully support CL to be a DTH reaction. However, patch testing may not be an adequate tool for detecting intra-oral elicited DTH reactions. As mercury can not directly be recognised by the immune system as T cells are limited to identify peptides derived from protein antigens (Romani *et al.* 1989), mercury has to react with a carrier protein, present in the oral tissue. Conformation changes to this protein, created by mercury, will be perceived as non-self by appropriate T cells. As mercury are conjugated to a specific carrier-protein to be recognized, it may be essential that the same carrier-protein is provided by the skin as in the oral mucosa. This reasoning may explain the high number of false-negative reactions to mercury using patch-testing on the skin. Intra-oral patch testing which may overcome this problem has been conducted (Axell *et al.* 1986, Luders 1987, van Loon 1984), but this technique is complicated to perform on a routine basi and it has been suggested that 5 to 12 times higher concentrations of the allergen is needed compared to a skin reaction. *In vitro* methods, using mononuclear blood cells, have been used to demonstrate sensitivity to mercury (Stejskal *et al.* 1994), but this method has not been accepted as more reliable than patch testing (Socialstyrelsen 1994).

Replacement of amalgam fillings to noble gold alloy or metal-ceramic crowns

A more complete healing response in the CL group was observed in patients who received noble gold casting alloys with or without acrylic veneers instead of metal-ceramic crowns ($p < 0.05$). It is possible that PFM crowns release substances from the interface between the alloy and the porcelain which negatively affected the healing response. However, this suggestion does not receive support from previous studies. Hensten-Pettersen for ex. reports that base metal alloys may cause less allergy problems than gold alloys (Hensten-Pettersen 1992), and palladium-type alloys have been suggested to be better tolerated than base metal and gold-based alloys (Mjör and Christensen 1993).

C. IMMUNOHISTOCHEMISTRY IN DIFFERENTIAL DIAGNOSIS OF ORAL LICHENOID TISSUE REACTIONS (IV)

As previously mentioned, oral lichenoid tissue reactions may only be differentiated into CL and OLP by means of its clinical appearance. Attempts have been made to differentiate between these lesions by histopathological methods (Bolewska and Reibel 1989), but without success. However, the superior healing potential after selective replacement of amalgam fillings and the higher frequency of cutaneous DTH reaction to mercury in patients affected by CL, support the concept that CL and OLP are caused by different antigens.

An effort to discriminate between CL and OLP by immunohistochemistry (IV) was based on a previous study from our laboratory (Simark-Mattsson *et al.* 1994). In the subepithelial infiltrate of OLP, T cells which used the V β 3-gene segments for creation of TCR were found to be approximately 10 times more numerous than in the cellular infiltrate of oral candidosis. This observation suggested that OLP may be instigated by a superantigen and, thus, the analysis of the number of T cells which used the V β 3-gene segments for creation of TCR may provide a basis for a discrimination of CL and OLP. This was the rationale behind the examination of the frequency of different T cell V-families in the subepithelial infiltrate of OLP and CL.

When different antibodies were used to show the expression of V β 3 T-cell families in OLP and CL lesions, an increased clonal expansion was found in both lesions when antibodies from clone LE89 were used. Antibodies from clones 8F10 and CH92 did not reveal any clonal expansion and similar figures as in peripheral blood were observed (Choi *et al.* 1989). These differences may be explained by a discrepancy of the antibodies to recognize different segments of the variable chain of the TCR or by an unspecific binding

of antibodies from clone LE89. The latter is supported by the fact that the expansion of the V β 3 T cell family almost disappeared when the blocking agents were changed from milk to rabbit serum. Staining with V β 3 (clone LE89) also showed a vague and undistinct pattern, while staining with V β 3.1 (clone 8F10) gave a distinct and clear staining pattern which was unaffected by different blocking procedures. The analysis of the frequencies cells expressing V α 2 and V β 5a did not reveal any difference between OLP and CL. Thus, it was not possible to discriminate between OLP and CL by immunohistochemical staining for the V-families examined.

The results of this study support the view that a lichenoid tissue reaction constitute a reaction pattern virtually independent of the nature of provoking antigens. The implication is that studies of the reaction pattern *per se* will not significantly contribute to our knowledge about the etiology of different lichenoid tissue reactions. Thus, CL and OLP, although released by different antigens, may not be discriminated by analysis of potential differences in the cellular reaction pattern.

D. EFFECTS OF RESIN COMPONENTS AND ROOT CANAL SEALERS ON IMMUNOCOMPETENT CELLS (V, VI)

In study V and VI components of resin composites and eluates of root canal sealers were tested in a con-A-driven proliferation of both spleen cells and purified T-lymphocytes co-incubated with pulpal cells. The results of these experiments suggest that the tested materials, at both toxic and subtoxic concentrations may alter the functional capacity of immunocompetent cells *in vitro*.

Immunosuppression by resin components and root canal sealers

With exception of CAMP, several of the resin components (UDMA, bis-GMA, TEGDMA, BPA, GMA, DHEpT, BGE-BPA and E-BPA) showed a concentration-dependent inhibitory effect on mitogen-driven proliferation of both spleen cells and T-cells, the latter activated by pulp cells. When pulp cells or T-cells were pre-treated with GMA, it was observed that proliferation was significantly decreased when T-cells but not pulpal cells were pre-treated ($p < 0.05$). This difference was not seen for the other materials employed (bis-GMA, UDMA and CAMP).

As with the resin components, the proliferative responses were suppressed with eluates of AH26, Grossman's sealer and Endométhasone in a concentration-dependent fashion in both spleen cell and pre-treated pulp cell assays. ID₂₀-values gave the following range according to cytotoxicity; AH26>Endométhasone>Grossman's sealer>Apexit (Table 3),

which agrees with results obtained from other cytotoxic tests (Beltes *et al.* 1995, Kersztesi and Kellner 1966, Nakamura *et al.* 1986, Wennberg *et al.* 1983). The calcium hydroxide based material Apexit, produced no cytotoxic effects, a finding which is similar to what has previously been reported (Beltes *et al.* 1995, Matsumoto *et al.* 1989). However, there are reports of cytotoxicity elicited by calcium hydroxide based materials (Spångberg 1969, Zmener and Cabrini 1987). These findings may be explained by the high pH provided by this type of material. When the pH is kept neutral by buffers in extraction solutions or culture media, this adverse *in vitro* effect is abolished (Hanks *et al.* 1983).

Table 3. ID₅₀s for resin components in $\mu\text{mol/L}$ and ID₂₀s for Extracted Root Canal Sealers (ERCS) (Dilution of extract in %)

| | Splenic Lymphocytes DNA Synthesis 24 h | Pulp Cells + T-lymphocytes DNA Synthesis 24 h |
|----------------------------------|--|---|
| <u>Resin components</u> | | |
| bis-GMA | 10.3 | 15.3 |
| TEGDMA | >50 | NA |
| CAMP | stim. only | stim. only |
| DHEpT | 760 | 500 |
| UDMA | 12.6 | 5.4 |
| GMA | 6.7 | 16.7 |
| BPA | 95 | NA |
| BGE-BPA | >25 | NA |
| E-BPA | 42.9 | NA |
| HDDM | >50 | NA |
| <u>ERCS</u> | | |
| AH 26 Fresk material | 1 | 2 |
| AH 26 Solid material | 5 | 13 |
| Endométhasone Fresh material | 2 | 2 |
| Endométhasone Solid material | 8 | 3 |
| Grossman´s sealer Fresh material | 16 | 14 |
| Grossman´s sealer Solid material | 10 | 15 |
| Apexit Fresh material | stim. only | 20 |
| Apexit Solid material | no effect | 20 |

It is tempting to speculate that suppression of immunocompetent cells elicited by dental materials, such as RCS and resin composites, may enhance the potential for bacterial injury to the dental pulp and periapical tissues. Thus, increased incidence of and severity of infections of these tissues are justifiable concerns following exposure to immunotoxic chemicals.

Fresh and solid material

The difference in cytotoxicity between extracts from fresh and solid material was evidenced by the AH 26 in the pulpal assay ($p < 0.01$). The results are similar to what has previously been reported (Matsumoto *et al.* 1989, Munaco *et al.* 1978, Spångberg 1981), suggesting that compounds from freshly mixed materials are more diffusible than those of set materials. The higher cytotoxic effect of freshly mixed material emphasizes the need for an appropriate mixing procedure as an incorrect powder/liquid ratio may produce a more toxic eluate.

Immunostimulation by resins and root canal sealers

It can be concluded from these studies that both resin components and ERCS have similar cytotoxic profiles. However, in addition to their suppressive effects, Grossman's sealer, Endométhasone, UDMA, bis-GMA, TEGDMA and BPA stimulated spleen cells to proliferation at low concentrations. Similar effects have been noted when low concentrations of components derived from Gram negative bacteria were used in a similar assay of spleen cells (Yoshida *et al.* 1995). In addition to antigen presenting cells and T-lymphocytes, the spleen also contains B-lymphocytes and it is possible that these cells were responsible for the observed increase in the proliferation rate.

A stimulatory effect was particularly noted for CAMP and this substance may have a mitogenic effect also under *in vivo* conditions. Further studies have to be conducted to pinpoint the exact nature behind this apparent effect.

Testing with spleen cells turned out to have several methodological advantages over the T-lymphocyte/pulp cell system. First, preparation of cells is much less time consuming and more simple to conduct than the more laborious purification of T-lymphocytes and preparation of pulpal cells. Second, spleen cells are more readily available and the number of animals which has to be used is certainly far less. However, the use of two separate cell entities that are functionally collaborating, offers the advantage of studying adverse effects on separate cells. In addition, the pulpal cell assay may be used to provide a more detailed information on the cells which may be the primary target for the components released from dental materials.

Test for DTH-reaction with subcutaneous implants

In order to test the ability of inducing a DTH-reaction, an implantation test was performed with subcutaneously implanted tubes containing the four RCS. All materials gave negative results following intradermal challenges. The current data support the view that RCS do not provide an enhanced risk to induce DTH-reactions (Gutierrez *et al.* 1986, Torabinejad and Kettering 1979). However, there are several case reports demonstrating local as well

as general DTH-reactions elicited by dental materials (Ebner and Kraft 1991, Hensten-Pettersen 1984, Hörsted and Söholm 1976). There are also experimental observations, using the Guinea pig maximization test (GPM-test), which demonstrate that both AH 26 and Endométhasone are able to induce a positive a DTH-reaction (Hensten-Pettersen *et al.* 1985). The different results may be due to methodological differences. In a GPM-test for example, the animal is repeatedly exposed to the allergen and in order to maximize the skin response, adjuvants and other chemicals are added.

MAIN OBSERVATIONS AND CONCLUSIONS

The observations made in this thesis support the view that potential adverse effects have to be analyzed by different methods which follow formalized and harmonized standards to provide essential information about the biocompatibility of dental materials. Thus, information about possible risks have to be based on extrapolation of results from both experimental tests *in vitro* and *in vivo*, and from studies of documented clinical side effects (Mjör 1992). Both the dental and the medical communities have a joint responsibility to convey obtained knowledge to the society through a sound and scientifically based information to minimize unjustified concerns on adverse effects related to dental materials.

- * A psychiatric diagnose was more common among the index group patients as compared to the controls. The prevailing symptoms were anxiety, asthenia and depression.
- * Somatic diseases were found to be more common among the index group patients compared to the controls.
- * Symptoms related to cranio-mandibular dysfunction were more frequently reported in the index group compared to the control group. The oral health status and the number of amalgam surfaces were similar in both groups.
- * Mercury levels in blood, urine and hair were similar among index cases and controls and far below critical levels reported for mercury intoxication.
- * There was no correlation between mercury levels and the severity of mental or somatic symptoms.
- * Lichenoid contact reactions showed a considerable improvement in 95% of the patients following selective replacement of dental amalgam fillings.
- * In patients with amalgam-associated lesions as a part of their oral lichen planus, only 63% showed an improvement following selective replacement. Oral lichen planus lesions in sites not in contact with dental amalgam were not affected.
- * The healing effect in patients who received noble gold alloy crowns was superior to the effect obtained with metal-ceramic crowns.

- * It was not possible to discriminate between lichenoid contact lesions and oral lichen planus by immunohistochemistry staining for different V-families.
- * The proliferative response of spleen cells and pulpal cells was inhibited in a concentration-dependent fashion when incubated with several of the tested resin components and eluates of root canal sealers.
- * At low concentration of some of the resin components and eluates of root canal sealers there was an increased cell proliferation compared to the control. One material, CAMP produced stimulation at all concentrations tested.
- * A delayed type hypersensitivity was not induced in rats following subcutaneous implants containing root canal sealers and challenge by ear injection.

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