In Vitro Evaluation of Human Pulpal Fibroblasts and L 929 Cells Exposed to Bacterial Proteins

Eleftheria Pissiotis
IN VITRO EVALUATION OF HUMAN PULPAL FIBROBLASTS
AND L 929 CELLS EXPOSED TO BACTERIAL PROTEINS

Eleftheria Pissiotis
D.D.S., Aristotelion University, Greece, 1984

A Thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Dental Science
at
The University of Connecticut
1990
Master of Dental Science

IN VITRO EVALUATION OF HUMAN PULPAL FIBROBLASTS AND L 929 CELLS EXPOSED TO BACTERIAL PROTEINS

Presented by
Eleftheria Pissiotis, D.D.S.

Major Adviser

Associate Adviser

Associate Adviser

The University of Connecticut
1990
TO MY PARENTS
ACKNOWLEDGMENTS

I would like to express my deepest appreciation to my major advisor and teacher Dr. Larz Spangberg for his guidance, support and valuable advice during my studies.

I am also very grateful to the members of my advisory committee, Dr. Kamran Safavi and Dr. Edward Kollar, for their assistance and constructive criticism during the preparation of the Thesis.

Special thanks to Dr. David Pashley for sending a sample of the spilL chamber, Dr. Paulette Tempro for the bacterial cultures, Dr. Arthur Hand and Mrs. Chris Pearson for the SEM photographs, Dr. Reza Kazemi for his artistic diagrams and Dr. Elizeu Pascon for his constant assistance in the laboratory.

I wish also to thank Mrs. Jenny Beaureng and Mrs. Sandra Pelletier for their assistance and patience during the typing of the manuscript.

I would certainly like to thank my friends Thanasis, John, Chris, and Ranie for their consultation and backing, as well as my friends in Greece for their support.

Finally I would like to thank the school for giving me this once in a lifetime opportunity.
PART 2 ................................................................. 35

Effect of Bacterial Extracts on Cells. Establishment of Dose response Relationships ............. 35

Materials and Methods ................................................. 35

a) Cells ................................................................. 35
b) Procedure ............................................................ 36
c) Cell counting .......................................................... 37
d) Incorporation of $^3$H thymidine ................................. 37
e) Scanning electron microscopy ........................................ 38

Results ................................................................. 38

Data Analysis ............................................................. 38

a) L 929 cells (murine fibroblasts) ................................. 39
b) Pulpal cells ............................................................ 40

PART 3 ................................................................. 44

The Moderating Effect of Dentin Membranes on the Cytotoxicity of Bacterial Proteins ............ 44

Materials and Methods .................................................. 44

a) The chamber .......................................................... 44
b) The incorporation of the dentin disks in the modified chamber ........................................ 45
c) Cytotoxicity of bacterial proteins after their passage through dentin membranes .............. 45

Results ................................................................. 46

DISCUSSION .............................................................. 49

CONCLUSIONS .......................................................... 65

REFERENCES ............................................................. 127
LIST OF TABLES

Table 1. Hydraulic Conductance of Dentin Disk.............. 116
Table 2. Bacterial Proteins Filtration through Dentin Disks.... 117
Table 3. Change in Hydraulic Conductance in Correlation with
          Proteins Retention........................................ 118
Table 4. Parameters for Comparison of Experimental Data Sets.... 119
Table 5. Effect of B. gingivalis W 83 Sonicated Extracts on
          L 929 Cells ............................................... 120
Table 6. Effect of B. gingivalis ATCC 33277 Sonicated Extracts
          on L 929 Cells ........................................... 120
Table 7. Effect of B. gingivalis W 83 Sonicated Extracts on
          Pulpal Cell Lines as Judged by \textsuperscript{3}H Thymidine
          Incorporation.............................................. 121
Table 8. Effect of B. gingivalis W 83 Sonicated Extracts on
          Pulpal Cell Lines as Judged by Cell Counting .......... 122
Table 9. Effect of B. gingivalis ATCC 33277 Sonicated Extracts
          on Pulpal Cell Lines as Judged by \textsuperscript{3}H Thymidine
          Incorporation.............................................. 122
Table 10. Thymidine Uptake of Pulpal Cells (PL\textsubscript{6} Fourth Passage)
          Challenged Through Dentin with B. gingivalis ATCC 33277
          (300 \textmu g/ml)............................................ 123
Table 11. Thymidine Uptake of Pulpal Cells (PL\textsubscript{6} Fifth Passage)
          Challenged with B. gingivalis ATCC 33277 (150 \textmu g/ml)
          Through Dentin.......................................... 124
Table 12. Thymidine Uptake of Pulpal Cells (PL₁₂ Fourth Passage)
Challenged with *B. gingivalis* ATCC 33277 (150 μg/ml)
Through Dentin .................. 125

Table 13. Inferred Bacterial Proteins Diffusion Coefficients
Through Dentin .................. 126
LIST OF FIGURES

Figure 1. Apparatus for dentin permeability measurements ........ 66
Figure 2. Dentin hydraulic conductance with PBS versus dentin hydraulic conductance with bacterial extracts ........ 68
Figure 3. Dentin hydraulic conductance before and after bacterial extracts filtration ......................... 70
Figure 4. Effect of Bacteroides gingivalis W 83 extracts on L 929 cells .................................................. 72
Figure 5A. Phase contrast micrograph of L 929 cells exposed to Bacteroides gingivalis W 83 ....................... 74
Figure 5B. Phase contrast micrograph of non-challenged L 929 cells (controls) ........................................... 74
Figure 6A. Scanning electron micrograph of L 929 cells exposed to Bacteroides gingivalis W 83 ....................... 76
Figure 6B. Scanning electron micrograph of non challenged L 929 cells (controls) ................................. 76
Figure 7. Effect of Bacteroides gingivalis ATCC 33277 on L 929 cells .................................................. 78
Figure 8. Effect of Bacteroides gingivalis W 83 extracts on pulpal cells (PL2) ................................. 80
Figure 9. Effect of Bacteroides gingivalis W 83 extracts on pulpal cells (PL4) ................................. 82
Figure 10. Effect of Bacteroides gingivalis W 83 extracts on pulpal cells (PL5) ................................. 84
Figure 11. Effect of *Bacteroides gingivalis* W 83 extracts on three pulpal cell lines (PL2, PL4, PL5), evaluated with thymidine incorporation ................. 86

Figure 12. Effect of *Bacteroides gingivalis* W 83 extracts on three pulpal cell lines (PL2, PL4, PL5), evaluated with cell counting ..................... 88

Figure 13. Comparison of the dose-response curves from all pulpal cell lines .................. 90

Figure 14A. Phase contrast micrograph of pulpal cells exposed to *Bacteroides gingivalis* W 83 .................. 92

Figure 14B. Phase contrast micrograph of non treated pulpal cells (controls) .................. 92

Figure 15. Effect of *Bacteroides gingivalis* ATCC 33277 extracts on pulpal cells ................ 94

Figure 16. Comparison between the cytotoxicity of the two strains of *Bacteroides gingivalis* extracts on pulpal cells .................. 96

Figure 17A. Phase contrast micrograph of pulpal cells exposed to *Bacteroides gingivalis* ATCC 33277 ............. 98

Figure 17B. Phase contrast micrograph of non challenged pulpal cells (controls) .................. 98

Figure 18A. Scanning electron micrograph of pulpal cells exposed to *Bacteroides gingivalis* ATCC 33277 extracts (x100) .......... 100

Figure 18B. Scanning electron micrograph of non challenged pulpal cells (x100) .................. 102
Figure 19A. Scanning electron micrograph of pulpal cells exposed to *Bacteroides gingivalis* ATCC 33277 (x500) .......... 102

Figure 19B. Scanning electron micrograph of control pulpal cells ......................... 102

Figure 20. Comparison of the cytotoxicity of *Bacteroides gingivalis* W 83 on L 929 cells and pulpal cells .......... 104

Figure 21. Comparison of the cytotoxicity of *Bacteroides gingivalis* ATCC 33277 on L 929 cells and pulpal cells .. 106

Figure 22. The Sykes-Moore chamber ......................... 108

Figure 23. The modified Sykes-Moore chamber .................. 110

Figure 24. The modified Sykes-Moore chamber in external and cross section view ....................... 112

Figure 24. A new microleakage model ......................... 114
INTRODUCTION

Pulpal inflammation has often been reported as a consequence of placement of dental restoration. The pulpal response has been attributed either to the direct chemical toxicity of the restorative material, or to the presence of bacteria in the contraction gap often present between the restoration and the cavity walls.

A number of studies pointed to the toxicity of the restorative material as the major cause of pulpal damage (Mitchell et al., 1962; Langeland et al., 1966; Stanley et al., 1967; Mjor et al., 1977). Brannstrom and Nyborg (1971, 1972) proposed that colonization of bacteria or accumulation of bacterial products under fillings are the principal causes of pulpal inflammation. Many studies (Brannstrom and Nyborg, 1973; Qvist, 1975; Skogedal and Ericksen, 1976; Mejare et al., 1979; Bergenholtz et al., 1982) confirmed a correlation between bacteria beneath restorations and inflammatory changes in the pulp.

Animal experimentation (Bergenholtz and Lindhe, 1975; Bergenholtz, 1977, 1981) offered convincing evidence to support the concept that products of dental plaque can be detrimental to the pulp. Extracts from human dental plaque deposited on freshly cut dentin of monkey teeth created an acute inflammatory response within a period of 8 to 32 hours. In addition, topical application of a non-toxic immunogenic substance induced inflammatory reactions in the dental pulp of monkeys immunized with this substance (Bergenholtz 1977). These studies demonstrated that freshly cut dentin does not serve as a protective barrier to the pulp.
Microorganisms or their metabolites can initiate inflammatory reactions in the pulp directly or indirectly through immunological mechanisms.

Penetration of biologically active substances through dentin shows that dentin is not impermeable. Scanning electron microscopic analysis (Garberoglio and Brannstrom 1976; Thomas and Carella 1983) has shown that dentin covering the dental pulp is penetrated by numerous tubules, and permitted calculation of their density and dimensions. The dentinal tubules provide the pathway by which bacteria and their products may reach the pulp. The phenomenon has been studied extensively in vivo and in vitro (Olgaert et al., 1974; Outwaite et al., 1976; Pashley, 1979, Pashley et al., 1981b); yet, no conclusive description exists of the transport mechanism(s) involved.

The difficulty in differentiating between chemical or microbiological causes of pulpal inflammation in vivo, motivates the need for well designed in vitro studies. Such experiments provide an attractive alternative, since they offer a controlled environment in which cause and effect relationships can be elucidated. There is, however, poor correlation between in vivo and in vitro methods mainly due to the fact that in vitro models cannot exactly simulate the in vivo conditions. One of the shortcomings has been the absence of a dentin interface, that acts as a barrier to the transport of toxic substances. Despite the fact that the function of dentin as a barrier to chemical toxic substances has been widely studied (Meryon 1981, 1984; Hume 1984; Hanks et al 1988; 1989), little is known about the transport of bacterial byproducts through dentin. In addition, even the direct effect of
bacterial byproducts on pulpal cells is largely unknown. These facts motivated the present investigation, with the following objectives.
OBJECTIVES

The objectives of the present study are:

1) to study the filtration of bacterial byproducts through dentin.

2) to investigate the direct effect of bacterial byproducts on different cell types using various techniques. Tritium labelled thymidine and cell counting will monitor cell function. Light and scanning electron microscopic analysis will be used to correlate morphological changes with the results from the quantitative evaluation.

3) to compare the responses of different human pulpal cell lines and a heteroploid cell line (L 929 cells) when exposed to the bacterial byproducts.

4) to compare cell responses of various pulpal cell lines to bacterial byproducts, and

5) to develop an in vitro model for the study of the moderating effect of dentin membranes on the cytotoxicity of bacterial extracts.
REVIEW OF THE LITERATURE

Biomaterial Evaluation In Vivo

Usage tests have often been applied to the study of the biological effects of dental restorative materials. The term "usage test" refers to an investigation of the effects of a material used in a manner identical or similar to its intended clinical application (Mjör, 1985). The pulpal response is evaluated histologically after the placement of the material in non-carious teeth of experimental animals or humans.

Many variables are involved in such experimentation, that may lead to poor reproducibility and reliability. A partial list of such variables include: the depth of the experimental cavity, the technique for cavity preparation, the uncertainty of the pulpal conditions at the insertion of the filling, and the host response (Tyas and Browne, 1977; Qvist and Stoltz, 1982).

Early reports from usage tests performed by Brannstrom and Nyborg (1971, 1972, 1973) indicated that the presence of bacteria correlated with pulp reactions under deep restorations of silicate cements and composite resins. In experiments where bacterial invasion was prevented, no inflammatory reactions of the pulp were reported even when known toxic materials were used. Bacterial growth in the contraction gap between the restoration and the cavity walls has also been confirmed by other researchers (Skogedal and Ericksen, 1976; Mejare et al., 1979; Bergenholtz et al., 1982). It was concluded that, although certain materials were toxic per se and initially could cause superficial pulpal...
irritation, persistent pulpal inflammation was the result of bacterial or bacterial byproducts penetration to the pulp through the remaining dentin.

In vivo experimentation pointed to the potential capacity of the pulp to survive injury by any restorative material in the absence of bacteria. Cox et al. (1987) showed that the pulp may produce new dentin matrix even in direct contact with a restorative material if no oral contamination could occur through leakage. Various materials such as silicate cements and zinc phosphate cements which were known to be very toxic were placed directly on the dental pulps of monkeys. Dentin matrix formation was observed when the surface of the restoration was sealed with ZOE cement. It was suggested that bacteria play a more important role in the development of pulpal disease after restoration than the restorative material itself. Furthermore various materials categorized as toxic when tested on germ-free animals elicited minimal pulpal responses (Watts and Patterson, 1987).

**Microleakage as the Major Cause of Pulpal Disease**

A common experimental model was used in all studies that demonstrated microleakage of bacterial byproducts as the major cause of pulpal inflammation after restoration (Brannstrom and Nyborg, 1971, 1972, 1973; Qvist and Qvist, 1977; Qvist 1980; Bergenholtz et al., 1982; Cox, 1987). Deep conventional class-V cavities were prepared in intact teeth of human beings or subhuman primates where the restorative material of interest was applied. As ZOE cement provides a very good cavity seal
preventing microleakage from being a contributing factor to pulpal inflammation, it was used in the outer part of the cavity as a mean to achieve a bacterially-tight seal (Brannstrom et al., 1979; Bergenholtz et al., 1982; Cox, 1987). At the end of the experimental period the teeth were evaluated histologically. These studies showed that microleakage correlated positively with pulpal irritation. In teeth where a ZOE cement was used for surface seal, little or no inflammation could be diagnosed. These ZOE cement fillings are placed in the superficial area of the cavity and therefore largely in the enamel layer. Furthermore the transport of eugenol through dentin is minimal (Hume, 1984). Consequently, it is unlikely that the use of eugenol would have an antibacterial action deep into the dentin (Hume, 1988). Thus, the absence of inflammation was attributed to the tight seal that prevented bacterial penetration.

Bacteria may either be present on the cavity walls before restoration or they may enter from the oral environment due to contraction or poor adaptation of the restorative material. Since aseptic conditions are usually maintained in modern restorative dentistry, it is not likely that significant numbers of bacteria are trapped during the restorative procedure. Brannstrom and Nyborg (1973), showed that even if the bacteria are eliminated before the placement of the restoration, bacterial colonization on the cavity walls develops within 4 weeks.

In order to obtain more information regarding such bacterial growth, Bergenholtz et al. (1982) placed a filter paper disk on the floor of the cavity beneath the restoration. Bacteria on the filter were
identified by microbiological analysis. Black pigmented *Bacteroides* and *Fusobacterium nucleatum* were observed in most of the samples.

Recent observations by Mejare et al. (1987) tend to support these findings. These investigators prepared, in an aseptic manner, class V cavities in human teeth. The preparations were checked microbiologically by culturing samples from the cavity floors. Composite restorations were placed after etching of the enamel surface margins of the cavities. After extraction, the teeth were split and samples from the floor of the cavity were re-cultured. The isolated bacteria from the bottom of the cavity were all common dental plaque bacteria.

The inherent difficulties in controlling microleakage, the need for human beings or subhuman primates, the extensive laboratory processing and cost of these experiments are some of the drawbacks of the *in vivo* experimentation. Therefore the development of different and more suitable types of experimentation for the evaluation of restorative materials is appropriate.

**Biomaterial Evaluation In Vitro**

*In vitro* methods have several desirable features. They offer better control of experimental variables, are less expensive and can often be performed in a short time period. Furthermore, they eliminate the use of animals and, if properly done, they permit greater reproducibility between laboratories (Browne, 1985).

Evaluation of biomaterials *in vitro* has been widely accepted and guidelines for their evaluation have been developed (ADA, 1979;
FDI, 1980). Most in vitro studies have been performed in cell cultures using established cell lines.

One of the most widely used methods of measuring cytotoxicity, is the "agar overlay method" as described by Guess et al (1965). According to that method, a confluent monolayer of vital-stained mouse fibroblasts (L 929) is established in a Petri dish and subsequently covered by a layer of nutrient agar. The test material is placed on the top of the agar and its cytotoxicity is assessed by the size of the zone of discoloration or cell lysis that can be observed in the cell monolayer. Although the method can be exact, reproducible and easy to perform, it has the disadvantage that it is applicable only to materials whose toxic components are able to diffuse through agar. Furthermore to extract useful information, it is also necessary to know the agar diffusion rate of the toxic substances. Due to these limitations, extrapolation of this in vitro results to in vivo expectations is not recommended, since critical in vivo parameters - such as the presence of dentin - are missing. Nevertheless, the agar-overlay method is sometimes very useful in obtaining basic information that explains in vivo phenomena (Schmalz, 1988).

Spångberg (1973) described the "chromium-release method" for the measurement of cytotoxicity of dental materials. The method is based on the phenomenon of cell protein leakage through the cell membrane as a consequence of cell damage. When vital target cells are labelled with $^{51}$Cr, the label is noncovalently bound to cell proteins and other cell constituents. Exposure to toxic biomaterials can cause cell damage and/or cell death. The subsequent lysis of the cells results in the
release of labelled cellular proteins which can be measured objectively. This measurement can serve as an indicator of cell damage. Provided that cell damage is proportional to the actual toxicity of the material, the method offers an objective, fast and effective means of in vitro evaluation.

Tyas (1977) introduced another in vitro model of toxicity testing of restorative materials, that was intended to simulate their clinical use. The test material was separated from the target cells by a membrane filter and a thin slice of dentin. Absence or decrease of enzyme activity (acid phosphatase or succinic dehydrogenase) in the cells are used as indicators of cytotoxicity of the material. Affected cells appear as unstained or less stained zones which can be evaluated macroscopically.

The "Millipore-filter method" first described by Wennberg et al (1979) was a modification of the previous method (Tyas 1977). A confluent cell monolayer is grown on a Millipore filter with medium and later covered by a semisolid agar medium. When solidified, the agar-filter system is inverted. Thus, the cells rest on the agar surface with the Millipore filter on the top. The test material is then placed on the filter for the designated experimental time after which the filter with attached cells is incubated for succinate dehydrogenase activity. Due to the close contact between the cells and the toxic material in this method, the test materials have a good chance to affect the cells through the pores of the filter. This method was not only used as a rapid screening test, but has served also as a model for some later
developments (Meryon 1981, 1984; Meryon et al., 1982; Meryon and Jakeman, 1986).

Meryon (1981) modified this *in vitro*-simulating model by incorporating a layer of compacted dentin powder between the material and the filter. They tested materials with and without the dentin interface and showed that the cytotoxic effect recorded was less in the presence of dentin powder. A number of materials such as ZOE cements, zinc-phosphate cements, and silicate cements were evaluated with that method.

**Absence of Dentin in In Vitro Cytotoxicity Models**

Most of the generally used *in vitro* methods did not take into consideration that *in vivo* restorative materials are not in direct contact with the tissue, but are separated by a layer of dentin. The lack of such a barrier of residual dentin in most *in vitro* assays may lead to the recording of unrealistically high levels of cytotoxicity.

In the "Millipore-filter method", the filter was designed to provide simulation of the dentin interposed between the material and the pulp. A similar effect may be considered for the agar interface in the agar overlay method. The frequently reported lack of correlation between *in vitro* and clinical tests indicates that most likely these artificial barriers do not adequately simulate dentin. It has been suggested (Hume, 1988; Pashley, 1988) that the unique chemical composition and microstructure of dentin dictates the use of dentin itself in cytotoxicity experiments *in vitro*. 
The significance of the protective qualities of the dentin has been shown by Hume (1984) who elegantly clarified the long standing paradox regarding the toxicity of eugenol. ZOE cement has a beneficial therapeutic effect when applied in dental cavities in vivo, but it is highly toxic when applied directly to soft tissue or to cells in culture. ZOE mixtures prepared with tritium-labelled eugenol were placed over dentin in human cavities. The teeth were mounted in a cylinder in such a way that saline was bathing the pulpal surface of the crowns. It was demonstrated that the presence of dentin decreases the passage of eugenol to the pulp by a thousand fold. At this concentration, eugenol is not cytotoxic; it can however, inhibit sensory nerve activity, prostaglandin synthesis, and vasoconstriction (Hume, 1988). Since eugenol is released by hydrolysis of ZOE cement, the concentration of available eugenol in the dentin-ZOE interface, is dependent on the presence of water in the area. In vitro experimentation showed that the floor of the dentin cavity, occupied by tubules filled with fluid is only between 1% and 22% of the whole surface area (Pashley, 1985). These data suggests that the absence of a dentin interface between the material and cells would render any in vitro toxicity evaluation of ZOE intendent as a cavity filling material of little clinical value because the evaluation would significantly overstate the problems (Hume, 1985).

Since dentin was usually absent in older in vitro models, it was generally suggested that in vitro techniques are suitable only for screening purposes of restorative materials. The information provided from all the presently used in vitro models was restricted to the
cytotoxic performance of the materials ignoring microleakage. In vitro models measuring microleakage are described in the next section.

In Vitro Methods of Studying Microleakage

Microleakage is defined as the passage of bacteria and their products as well as fluids from the oral environment through the gap between the cavity wall and the restorative material (Kidd, 1976; Bauer and Henson, 1984). Among the factors that contribute to microleakage are the inadequate sealing properties of the material, the improper restorative technique, and the presence of interfacial space (the space that exists between tooth structure and the restorative material, base or liner) due to a lack of adhesion (Bauer and Henson, 1984). The importance of microleakage to dental health, and the methods used for its detection have been reviewed by many investigators (Going, 1972; Jodaikin, 1981; Kidd, 1976; Browne and Tobias, 1986). Microleakage has been associated with various clinical conditions such as recurrent caries, tooth discoloration, thermal hypersensitivity and pulpal damage.

In vitro methods to detect and trace microleakage included the use of organic dyes, radioactive isotopes, compressed air, artificial caries, and penetration of bacteria. The use of organic dyes is one of the oldest methods for the detection of microleakage (Grossman, 1939). In this method, a restored tooth is immersed in a dye solution. After sectioning, microleakage is evaluated by assessing with various methods the extent of dye penetration. Although extensively used, the validity of this method has never been established, since there are parameters
(such as entrapment of air) that have never been taken into consideration (Spangberg et al., 1989). Air pressure for the tracing of leakage around fillings was first used by Harper (1912). Pickard and Gayford (1965) used the air pressure method in which air was forced through potential microleakage areas under water. The production of bubbles was the indication of leakage. Granath and Svenson (1970), extended the air pressure method and created a new apparatus for registering in vitro leakage. They showed that, although the air pressure method cannot be quantitative, it is suitable for qualitative comparisons. Penetration of radioactive $^{45}$Ca at restoration-dentin margins, as shown by autoradiographs, has also been used for microleakage measurements (Phillips et al., 1961).

The artificial caries technique, first described by Silverstone (1967), has been extensively used for leakage measurements. An acidified gelatin gel was used to produce artificial caries around restorations. The method has the advantage that microleakage can be linked directly to the development of an artificial lesion. Quantitation is possible by measurements of the penetration of the lesion (Kidd, 1976).

In an attempt to use a method which would more likely mimic the clinical situation, researchers have used bacteria as tracers of microleakage. Fraser (1929), tested cements and filling materials with a method that was later refined by Mortensen et al. (1965). Bacteria were placed over restorative materials glued between the walls of glass tubes. Turbidity of the underlying sterile broth after passage of bacteria through the material-glass space was a sign of microleakage. Mortensen et al. (1965) employed a tooth model. They isolated the crown
of the tooth with its restoration from the root using a plastic tube sealed with resin. Contaminated broth was placed over the crown, and sterile broth in contact with the root. Turbidity of the sterile broth was again the indication of microleakage. Seltzer (1955) studied the bacterial penetration through restoration margins by using two color-producing microorganisms (\textit{B. globigii}, and \textit{S. macescens}). He reported that these microorganisms did not penetrate the restoration-dentin interface, unless the teeth had undergone thermal cycling.

The literature that deals with the phenomenon of microleakage of restorative materials is limited to the study of the material-cavity interface. However, bacteria or their byproducts leaking through the interface have to reach the bottom of the cavity, bypass the smear layer, and travel through the dentinal tubules in order to cause pulpal injury. The smear layer is a layer of organic matrix covering the dentin surface after the cutting procedure. Since the methods of cavity preparations vary, the nature and the thickness of the smear layer is variable. Elimination of the smear layer is usually accomplished by acid etching or chelation of the dentin surface. The interactions between bacteria and the smear layer, have been studied repeatedly. Olgart et al. (1974) in \textit{in vitro} and \textit{in vivo} experiments showed that acid-producing microorganisms are capable of dissolving the smear layer, and entering into the dentinal tubules. Williams and Goldman (1985) proved that the presence of the smear layer did not inhibit the passage of \textit{Proteus vulgaris} through the dentinal tubules, although it delayed it. Finally, Meryon et al. (1986) showed that \textit{Pseudomonas aeruginosa} is capable of penetrating unetched dentin slices.
As discussed previously, present \textit{in vitro} models either evaluate the cytotoxicity of a material or its microleakage separately. Incorporation of the dentin interface could, in principal, provide a means of measuring the combined effects of microleakage and cytotoxicity and clarify the interaction which results in pulpal disease. To our knowledge such a model is not available; nor has it yet been proposed.

\textbf{Dentin as a Permeable Barrier}

Transport of toxic stimulants of chemical or bacteriological origin to the pulp, is regulated by the continuous outward flow of dentinal fluid. Pashley (1979) discussed the important balance between the rate at which pulpal blood flow can carry these solutes away from the pulpal ends of the tubules. He demonstrated that reduction in pulpal blood flow can result in an increase in concentration of toxic substances in the pulpal interstitial fluid. It is now established that a fully functioning pulpal blood flow can be very efficient in keeping exogenous substances at a low level at the dentin pulp interface. Rapid dentinal fluid movement in the dental tubules has also been implicated in the etiology of dentin sensitivity (Brännström et al., 1967). The authors suggested that "transmission of pain-producing stimuli to the pulp may have a hydrodynamic mechanism".

Transport of substances through dentin can be described in terms of two basic laws:

1. Darcy's Law (Scheidegger, 1960) for bulk fluid transport through a porous medium.
\[ \frac{\Delta P}{L} = \mu \frac{Q}{k A} \]  \hspace{1cm} (1)

where: \( \Delta P \) = Pressure difference

\( k \) = Darcy permeability of the porous medium

\( \mu \) = Viscosity of the fluid used

\( L \) = Thickness of the porous medium

\( Q \) = Volumetric fluid flow rate

\( A \) = Frontal area available for fluid transport

The combination \( k/\mu \) is termed "permeability constant" (\( K',\text{Scheidegger, 1960} \)), and it is indicative of the permeability of a porous medium to a particular fluid. The Darcy permeability \( (k) \), is a geometrical characteristic of the porous medium microstructure. It has units of area \( (\text{cm}^2 \text{ in CGS system}) \), thus it can be thought of (in a loose sense), as a measure of the available "pore" area for flow. For a particular porous medium model, \( k \) can be computed from the geometrical parameters of the model. The simplest porous media model that may describe dentin is that of a bundle of parallel straight capillaries.

The Poiseuille-Hagen Law for a single capillary (\( \text{Scheidegger, 1960} \)) illustrates the important variables that influence convective transport (bulk fluid movement).

\[ Q = \pi \frac{\Delta P \ r^4}{8 \mu L} \]  \hspace{1cm} (2)
where:

\[ Q = \text{Volumetric fluid flow rate} \]
\[ \Delta P = \text{Pressure difference} \]
\[ r = \text{Radius of capillary (Functional tubule radius)} \]
\[ \mu = \text{Viscosity of the fluid} \]
\[ L = \text{Length of the tubule} \]

For \( N \) capillaries the second part of the equation is multiplied by \( N \). From equations (1) and (2) one can derive:

\[ K' = \pi r^4 N / 8 \mu A \]  \( (3) \)

While \( k \) and \( K' \) provide a more fundamental description of a porous medium, some authors (for a review see Pashley 1990), use another parameter (the hydraulic conductance \( L_p \)) to describe convective fluid flow across dentin.

\[ L_p = Q / \Delta P A \]  \( (4) \)

where

\[ Q = \text{Volumetric fluid flow} \]
\[ A = \text{Frontal area available for fluid transport} \]
\[ \Delta P = \text{Pressure difference} \]

\( L_p \) and \( K' \) are connected with the following formula:

\[ K' = L_p L \]  \( (5) \)
derived from equations (1) and (4).

2. Fick's Law of diffusion (see e.g. Probstein, 1989). Fick's Law of diffusion illustrates the important variables that control diffusive transport of fluid solutes across dentin:

\[ J = D_{\text{eff}} \left( \frac{\Delta c}{L} \right) \quad (6) \]

where:

- \( J \) = Diffusive flux of solute
- \( D_{\text{eff}} \) = Effective diffusion coefficient of solute
- \( \Delta c \) = Solute concentration difference between the two sides of dentin
- \( L \) = Thickness of dentin

Both modes of substance transport through dentin (bulk fluid movement and diffusive transport) are related to the available open-pore space that for dentin, is affected by the tubule diameter and number per unit area. Since both of these parameters increase closer to the pulp (Garberoglio and Brannstrom, 1976), substance transport becomes easier as the pulp chamber is approached (Pashley, 1978a). Factors influencing the tubule diameter, include the presence of odontoblastic process, the presence of collagenous fibers in the tubules and the sheet-like lamina limitans that lines the tubules (Thomas and Carella 1983; Thomas 1984). Michelich et al. (1978) showed that the functional tubule radius of Eq. (2) is about 5-40% of the reported anatomical radius determined by electron microscopy.
Transport of fluids to the pulp is dependent upon many variables as one can appreciate from the above equations. A convenient classification might distinguish among tooth variables and fluid variables.

The thickness of the remaining dentin, the degree of tubule occlusion, and the presence of a smear layer are all parameters of the tooth that affect the permeation rate of the solute. The thicker the dentin and the more the dentinal tubules are occluded, the lower the permeation rate. The potential importance of the smear layer is based on its function to occlude the dentinal tubules. Removing the smear layer by etching in order to increase mechanical retention, or clean the surface, opens the tubules and facilitates passage of solutes. The fluid variables such as the viscosity, the concentration of the solute, the molecular size of the solid molecules, and their binding ability, can affect the fluid influx as well. All of these variables have been studied extensively in vitro (Outwaite et al., 1976; Pashley et al., 1977; Michelich et al., 1978; Reeder et al., 1978; Pashley et al., 1978 a,b; Pashley et al., 1981a).

Several techniques have been used to study dentin permeability. Although most of the present information has been obtained from in vitro studies, comparable in vivo studies are lacking. The few in vivo studies that have been published involve measurements of the motion of dyes (Lefkowitz, 1943), radioactive isotopes (Pashley et al., 1981c; Potts et al., 1985) and fluid across dentin (Pashley et al., 1981b). Pashley and coworkers (Pashley et al., 1981c) showed in experiments with dogs that permeation of $^{131}I$ in vivo and in vitro on the same teeth was very
similar. They suggested that the absence of the living odontoblastic process in the \textit{in vitro} experiments did not alter the permeation rate. It was suggested that permeation of larger molecules or lipid soluble substances might not be similar \textit{in vivo} and \textit{in vitro} due to other factors.

Dentin permeability was initially measured \textit{in vitro} by placing intact tooth crowns (isolated from the pulpal site) in radioactive buffer and checking the amount of isotope that was passing through its structure after the establishment of a pressure gradient (Smith and DeVicenzo, 1968). Although this protocol provided an estimate of both enamel and dentin permeability, it was used as a basis for future experimental models (Hume, 1984, 1985).

The most established model to study dentin permeability is by using disks of dentin clamped between two rubber O rings in a "split chamber device" (Outwaite et al., 1974). Both sides of the chamber are filled with a buffer solution which is filtered through the dentin specimen under pressure. The rate of fluid movement across dentin is measured by following the movement of an air bubble in a micropipette incorporated in the system (Reeder et al., 1978; Pashley et al., 1978 a, b). In the \textit{in vivo} measurements of dentin permeability specially designed chambers are cemented to dentin and connected via micropipette to the device providing the pressure. Similarly fluid movement was estimated by following the movement of the air bubble in the micropipette (Pashley et al., 1981c; Pashley et al., 1983, 1984). Quantitation of dentin permeability was expressed in terms of hydraulic conductance.
Recently Derkson et al. (1986) showed that the principles for the measurement of dentin permeability can also be applied to the measurement of microleakage. Leakage occurring around temporary filling materials (Cavit, IRM, TERM) was measured with a similar method (Anderson et al., 1988; Pashley et al., 1990). The fluid filtration method was also used in the measurement of microleakage of several retrograde filling materials (King et al., 1990; Yashimura et al., 1990).

Hanks et al. (1988, 1989) modified the model described by Pashley in order to study the cytotoxic effect of composites. These investigators acknowledged that with the present methods of cytotoxicity evaluation it is difficult to distinguish between chemical and microbiological (due to leakage) toxicity. An apparatus to simulate of the pulpal chamber was adapted from the "split chamber device". It permits a cell monolayer to be grown in the bottom of the chamber. Numerous composite materials were tested. The composite is placed over a dentin disk which is placed on the top of the chamber. Evaluation of the protein synthesis of the cultured cells is a measure of the cytotoxicity of the composites. It was concluded that dentin significantly attenuates the toxic effect of the material. The diffusion characteristics of phenol (a well known cytotoxic agent) were also studied. Again, the dentin significantly reduced the cytotoxic effect of phenol.

Hanks et al. (1988) suggested that the basic principle of this model can be used to assess the attenuating effect of dentin in transferring bacterial substances to the cell line provided that a
bacterial tracer with a known toxic effect is available. It is known from in vivo studies (Bergenholtz and Lindhe, 1975; Bergenholtz 1977) that bacterial byproducts pass through dentin resulting in pulpal disease. Although bacterial permeation through dentin was shown in vitro (Michelich et al., 1980; Meryon et al., 1986; Meryon and Brook 1990) and in vivo (Lundy & Stanley, 1969; Olgart et al., 1974; Mjör, 1977), the permeation of bacterial byproducts was never investigated in vitro. Furthermore, the in vitro toxicity of bacterial byproducts to cells of pulpal origin was not tested. There is, however, substantial evidence in the periodontal literature regarding the cytotoxic effect of bacterial byproducts to cells of periodontal origin as well as to various immortal cell lines.

Cell Exposure to Bacterial Byproducts

Experimentation with direct exposure of cultured cells to bacterial components has been published in the periodontal literature. Gingival fibroblasts or various immortal cell lines were exposed to different concentrations of sonicated plaque or bacterial byproducts. Their cytotoxic effect was evaluated: a) with the radiometric method; b) with the incorporation of labelled thymidine c) by cell counting, or d) with other methods.

Levine et al. (1974) showed that plaque extracts prevented growth of HeLa cells and suggested that inhibition of cell growth after exposure to toxic components of dental plaque may be associated with the incidence of periodontal disease. Further, HeLa cell cytotoxicity
studies with streptococcal extracts (Duguid et al., 1980; Duguid and Lyons, 1982) indicated that lower molecular weight components of the extracts stimulated cell growth but higher molecular weight components inhibited cell growth. These investigators suggested that $^3$H thymidine uptake of the cells over a period of 24 hours is a more effective method of evaluating toxicity of bacterial extracts compared with cell counting and $^{51}$Cr release. The effect of bacterial extracts on immortal cell lines was also studied by Van Steenbergen et al (1982) and Grenier and Mayrand (1985) on Vero cells. Fusobacterium nucleatum and black-pigmented Bacteroides were tested and their cytotoxicity was evaluated after staining the methanol fixed cells with Giemsa stain. Bacteroides gingivalis (B. gingivalis) was shown to be the most virulent of all bacteria tested as judged by this evaluation method based on cellular morphology.

Sonicated extracts from spirochetes were tested by Boehringer et al. (1984) on a murine fibroblast cell line (L 929 cells) and on human skin fibroblasts. A dose dependent toxic effect of the spirochetes extracts on fibroblast proliferation was shown by inhibition of $^3$H thymidine, $^3$H uridine, $^3$H leucine incorporation and also by cell counting. Inhibition of fibroblast growth after exposure to spirochete extracts in vitro was suggested to be associated with connective tissue destruction in periodontal disease.

The effect of extracts from bacteria known as periodontal pathogens was further studied on periodontal ligament fibroblasts and gingival fibroblasts in vitro (Stevens et al., 1983; Duguid 1985; Larjava et al., 1987; Larjava and Vitto, 1987). The extracts that were used originated
from *Actinobacillus actinomycetemcomitans* (A.a), *Capnocytophaga*, *
Fusobacterium nucleatun* (F.n.) and black-pigmented *Bacteroides*. Most
studies agreed that these extracts caused a significant inhibition of
cell growth and some indicated the exact protein dose where inhibition
starts. Larjava et al. (1977) and Larjava and Vitto (1987) studied the
effect of *Bacteroides* extracts on gingival fibroblast growth by
monitoring thymidine uptake and cell multiplication. Among all the
*Bacteroides* species tested (*B. gingivalis*, *B. assacharolyticus* and *B.
intermedius*), *B. gingivalis* extracts were the most potent growth
inhibitors while the rest showed only slight inhibitory effect. As a
matter of fact, small doses (0-10 μg/ml) of proteins from *B.
assacharolyticus* and *B. intermedius* showed DNA stimulation. Despite the
observed growth inhibition (in higher doses up to 100 μg/ml), the
fibroblasts appear viable with phase-contrast microscopy and by
cytological staining (May-Grunwald-Giemsal staining).

These investigators used many different lines of gingival
fibroblasts and concluded that each line responded differently to the
bacterial extracts. The inhibition of cell growth after exposure to *B.
gingivalis* (50 μg/ml) varied from 12% to 60% among the twelve cell
lines. Although the number of cell lines was considered small, it was
suggested that genetic heterogeneity of fibroblasts between the
individuals (that the gingival tissues were taken from) may account for
the different response.

Although useful information regarding the pathogenicity of
different bacterial extracts can be retrieved from the above studies,
comparison of the cytoxic effect of these extracts is difficult.
Differences in the cell line, in the dosage of the bacterial extract used, and in the evaluation method selected are responsible for the lack of clear cut dose-response effects in the literature. Recently, Stevens and Hammond (1988) attempted a comprehensive comparison of the effect of nine periodontal bacterial extracts on human gingival fibroblasts and suggested that *A. a., F.n.*, and *B. gingivalis* extracts are the most potent growth inhibitors.

Fibroblast reactions to bacterial products may account for the pulpal tissue destruction associated with different degrees of pulpal pathosis. Altered pulpal fibroblast function and proliferation can be the result of bacterial products reaching the pulp through the dentinal tubules. Although pulpal fibroblasts (animal and human) have been used in biocompatibility testing (Miller et al., 1976a; Munaco et al., 1978; Kawahara et al., 1979; Das 1981; Hanks et al. 1983), their response to bacterial irritants has rarely been investigated. Pinero et al. (1983) exposed human and bovine pulpal fibroblasts to different doses of *E. coli* endotoxin and studied synthesis of DNA collagen and glycosaminoglycans (GAG's). Their results indicated that low levels (5-125 µg/ml) of endotoxins actually stimulate cell division and connective tissue matrix synthesis which is in contrast to previous observations on the growth-inhibitory effect of endotoxin on L 929 cells (Singer and Button, 1979). Higher doses (625 µg/ml) inhibited cell division but continued to provoke collagen production in the human pulpal cell lines. Assuming that the results correlate with the clinical situation, these investigators considered the productive reactions to endotoxin as a defensive response. Except for this study the response of pulpal cells
to bacterial stimuli \textit{in vitro}, has not been tested. Although methods for isolation of cells from adult human or bovine dental pulp have been described in detail (Miller et al., 1976b; Levin et al., 1988), the function of pulpal cells \textit{in vitro} has been poorly investigated.
MATERIALS, METHODS, AND RESULTS

Outline

In order to study the toxic effect bacterial products may have on pulpal cells several aspects of their toxicity and their filtration through dentin must be studied. Therefore, three different experiments were performed.

In the first experiment, the role of dentin as a porous membrane was investigated. Using an established model, the hydraulic conductance of dentin was studied and its ability to selectively filtrate proteins of bacteria was calculated.

In the second experiment, the cells of interest (pulpal cells and L 929 cells) were exposed to different concentrations of bacterial extracts. The reaction of the cells was evaluated with two methods. The results obtained were used to establish dose response curves.

Finally, the cells were exposed to bacterial extracts through dentin in a specially designed chamber intended to simulate in vivo conditions. The effect on the cells was recorded and compared with the earlier established dose response curves. This comparison permitted an for estimation of the concentration of bacterial extracts that passed through the dentin.
PART 1

Permeation Rate of the Bacterial Extracts Through Dentin Barriers

The hydraulic conductance of dentin specimens was determined using phosphate buffered saline (PBS) and bacterial suspension as a filtrate. Changes in the concentration of the bacterial proteins as a result of filtration through dentin were recorded. A final filtration with PBS was performed to elucidate whether any alterations of dentin permeability are permanent.

Materials and Methods

a) Bacterial proteins

*B. gingivalis* ATCC 33277 and W 83 were cultured anaerobically on Schaedler broth (BDL), containing 8.1 g of pancreatic digest of casein, 2.5 g of peptic digest of animal tissue, 5.82 g of dextrose, 5.0 g of yeast extract, and 1.7 g of sodium chloride per liter of purified water.

Following incubation, the bacterial cells were harvested by centrifugation (9000 x g for 20 min), washed with Tris buffer and recentrifuged twice (9000 x g for 20 min). The washed cells were pelleted by a final centrifugation (4000 x g for 30 min). The pellets were lyophilized and stored at -20°C. For use the lyophilized bacteria were diluted with PBS and sonicated on ice with a Braun Sonic 1510 sonicator at 100 W output. Disruption of the cells was confirmed microscopically. The sample was centrifuged (11,000 x g for 90') and the
cell-free supernatant was kept for experimentation. The protein concentration of the supernatant was determined spectrophotometrically with the method of Smith et al. (1985), using albumin as a standard. Dilutions of proteins to various concentrations (10-250 μg per ml) were made with culture media (MEM) or PBS at the time of each experiment.

b) Evaluation of hydraulic conductance using PBS as a filtrate

Extracted non-carious human molar teeth which had been stored in saline were used. All teeth were used within a month of extraction. Each tooth was mounted with acrylic (Coldpac, The Motioid Co., Inc., Chicago IL) on a plexiglass base, and positioned in a Gillings-Hamco cutting machine. The enamel was removed in a plane parallel to the occlusal surface of the tooth. The second cut was made parallel to the first surface. The thickness of each disk was adjusted to approximately 0.4 mm. The smear layer created by the cutting was removed with 3 minutes of surface treatment with 0.5M EDTA. The treated disks were placed in a "split chamber device" which has been described in detail by Outwaite et al (Fig.1). The chamber was connected via polyethylene tubing to a 180 cm column of PBS supplying the hydrostatic pressure necessary to force the fluid towards the lower compartment of the "split chamber". A micropipette (25 μl Microcap) was interposed between the column and the chamber. A small air bubble was introduced in this capillary making it possible to follow any fluid movement through it. This fluid movement was compensated with the aid of a 2 ml micrometer microsyringe (Gilmont Instrument, Inc.). This compensation of fluid movement through the capillary permitted flow rate calculations. The
filtration characteristics of each dentin disk were expressed as hydraulic conductance ($L_p$). The effect of varying disk thickness can be eliminated using Darcy's Law to compute the "permeability constant" $K'$ (recall from Introduction p.19, $K' = L_p L$).

The pressure difference was equivalent to 180 cm PBS (which is equal to 180 cm $H_2O$) and the surface area was kept constant, by using the same O-rings (0.4mm in diameter) in the split chamber ($A = \pi d^2 / 4 = 3.14 \times 0.4 \times 0.4 / 4 \ cm^2 = 0.1256 \ cm^2$).

$L_p$ was expressed as $\mu l / \min \ cmH_2O \ cm^2$.

c. Evaluation of the hydraulic conductance using bacterial extracts as a filtrate. Determination of concentration changes.

The methods described under section b) were used. PBS was replaced by a suspension of bacterial extracts. Preliminary experiments regarding the cytotoxicity of $B. \ gingivalis$ ATCC 33277 extracts indicated that 200 $\mu g/ml$ (of protein concentration) had a strong inhibitory effect on cell growth in vitro. Since the purpose of the study was to assess whether bacterial byproducts can be transported through dentin, $B. \ gingivalis$ ATCC 33277 supernatant was diluted with PBS to a final concentration of 200 $\mu g/ml$. This replaced PBS in the hydraulic column. The pressure difference imposed by the column was again maintained at 180 cm PBS.

As fluid was exiting from the upper part of the split chamber, samples were collected with a 50 $\mu l$ Hamilton microsyringe (Hamilton Corp. Reno NE) and subjected to a protein determination assay (Smith et
al 1985). Thus, any changes in the concentration of proteins after filtration through the dentin barriers could be calculated.

Since all the parameters were held constant, each dentin disk served as its own control. $L_p$ values when bacterial extracts were filtrated ($L_{p2}$) were compared with the initial values ($L_{p1}$). Comparison using $K'$ values would give equivalent results, since thickness of each disk was kept constant.

d) Refiltration of the dentin disks with PBS

After the filtration of bacterial extracts through the dentin disks, the column was drained and washed with 1% NaOCl. The tubing as well as the micropipette were replaced with new ones. The dentin disks were flushed with PBS and subsequently stored in saline. The experiment was repeated with the same dentin disks after filling the hydraulic column with PBS. The hydraulic conductance ($L_{p3}$) of the dentin disks was remeasured under similar conditions as in the previous experiment. These values were compared with the baseline values obtained in the initial PBS filtration of the disks. $L_{p1}$, $L_{p2}$, and $L_{p3}$ values were compared statistically using the Mann-Whitney test (Zar 1984).

Results

The experimental data of the dentin permeability tests are summarized in Table 1. The results are expressed as hydraulic conductances ($L_p$)$_n$ as well as the permeability constants ($K'$)$_n$ for $n=1,2,3$. Subscript 1 refers to the initial experiments (PBS filtration), 2 to the
bacterial extracts filtration experiments, and 3 to the final experiments (PBS filtration) conducted to detect residual changes in dentin permeability.

A total of 10 dentin disks were used. Their thickness ranged from 0.3 mm to 0.55 mm with a mean of 0.379 mm and a SD of 0.075 mm. Each disk served as its own control in the three consecutive measurements. As seen from Table 1, $L_{p1}$ values had a mean of $3.52 \times 10^{-2} \pm 1.75 \times 10^{-2}$ μl/cmH20.min.cm$^2$. The mean $L_{p2}$ value is $2.09 \times 10^{-2} \pm 0.65 \times 10^{-2}$ μl/cmH20.min.cm$^2$. $L_{p2}$ is statistically significant lower than $L_{p1}$ (Mann Whitney: P<0.05). Figure 2 illustrates the correlation between $L_{p1}$ and $L_{p2}$.

The final measurements of hydraulic conductance ($L_{p3}$) had a mean of $3.74 \times 10^{-2} \pm 1.54 \times 10^{-2}$ μl/cmH20.min.cm$^2$ which is not statistically significantly different than the initial measurements ($L_{p1}$) (Mann Whitney, p<0.05). Figure 3 shows the relationship between $L_{p1}$ and $L_{p3}$.

The respective permeability constant values ($K'$) show the same trend as the hydraulic conductance values. $K'_2$ values are statistically significantly lower than $K'_1$ ones (Mann Whitney p<0.05) while $K'_3$ are not statistically significant different from the $K'_1$ values.

The retention of the bacterial extracts through dentin varied (Table 2). From the ten disks examined, four did not show any detectable protein retention. For the rest the retention varied between 22.5% and 75.5% with a mean of 40.3% and a SD of 18.4 μg/ml.

This part of the experimentation showed that dentin allows passage of $B. gingivalis$ sonicates (200 μg of protein/ml). The permeability of the dentin is significantly lowered during this filtration. The
percentage reduction in permeability does not have a one to one correspondence with the percentage retention of bacterial proteins (Table 3).
PART 2

Effect of Bacterial Extracts on Cells.

Establishment of Dose-response Relationships.

The purpose of this part of the study was to evaluate the toxicity to the cells of bacterial extracts. Different concentrations of bacterial extracts were used in order to establish a dose-response relationship. Incorporation of tritiated thymidine and cell counting served as the basic methods to evaluate cell function. Light microscopic as well as electron microscopic analysis were also used.

Materials and Methods

a) Cells

Pulpal fibroblasts were obtained from explants of pulps from extracted third molars. The extracted teeth were held in gauze saturated with 2% iodine potassium iodide. Soft tissue remnants were removed. In order to provide a fracture plane, a groove was made in each tooth with a diamond saw under running sterile saline. The tooth was cracked open with a sterile chisel. The pulp tissue was harvested aseptically and immediately placed in a Petri dish with culture medium supplemented with antibiotics (100 IU penicillin/ml and 50 µg streptomycin/ml). In the laminar flow hood, the explants were washed three times with complete medium and minced into small pieces. The pieces were transferred to a sterile tissue culture flask, left with a
thin film of culture medium for 20 minutes to improve attachment, and later covered with fresh culture medium. The flasks were immediately placed in the incubator at 37°C in a humidified atmosphere of 5% CO₂ - 95% air. After a period of 5-10 days the fibroblasts proliferated from the explants. At confluency, the cells were detached with 0.02% trypsin and transferred to 75 cm² flasks. Cells were used for the experiments after the third passage. Five pulpal cell cultures were used for the experimentation.

The murine fibroblast cell line (L 929) as well as the pulpal cell cultures, were cultured in Minimal Essential Medium (MEM, Flow Laboratories, Rockville, MD) supplemented with 10% serum (Hyclone Labs, Logan UT), antibiotics (100 IU penicillin/ml and 50 μg streptomycin/ml) and L-glutamine 2 mM.

b) Procedure

The cells were harvested with 0.02% trypsin in Ca++ and Mg++ free PBS, transferred to Petri dishes (35mm diameter) at a concentration of 100,000 cells/ml and grown in 2 ml of medium. The bottom of the Petri dish was covered by a Thermolox tissue culture coverslip (Lux, Nunc Inc, Naperville IL). After 32 hours of incubation, the medium was removed and replaced by new medium containing dilutions of the bacterial extracts at 10, 50, 100, 150, 200, and 250 μg/ml of protein. The effect on the cells was examined after an additional 24 hours of incubation. Cell counting and incorporation of tritiated thymidine were used for the evaluation of cell injury. Scanning electron microscopy of experimental and control cultures was performed in selected experiments. Each
experiment was run in triplicate. Experiments were performed with pulpal and L 929 cells and both strains of \textit{B. gingivalis} (W 83 and ATCC 33277). Dose-response curves were calculated for all cell lines.

c) Cell Counting

The experimental and control cell cultures were washed with PBS, detached with trypsin, transferred to cuvettes containing 10 ml of isotonic saline (Travenol Labs Inc, Deerfield IL) and counted with a Coulter counter (Coulter Electronics Ltd, Luton, Beds, LU3 3RH, UK). The number of cells counted in the experimental cultures was calculated as percentage of the controls.

d) Incorporation of $^{3}$H thymidine

$^{3}$H-thymidine incorporation was used in order to monitor the rate of DNA synthesis of the cells. The technique has been described earlier by Wennberg (1976). During the last 3 hours of experimental incubation, the culture medium was aspirated and the cells were pulsed with 3$\mu$Ci/ml of $^{3}$H thymidine (Specific activity 6.7 Ci/mmol, Dupont NEN, Boston MA) diluted in fresh culture medium.

At the end of the 3 hour period, the medium was withdrawn and the cell monolayers were washed 3 times with 5% ice cold trichloroacetic acid. After the last wash, the plastic coverslips were removed from the petri dishes, cut into small pieces and transferred to the scintillation vials containing tissue solubilizer (Protosol, NEN, Boston MA). Ten ml of scintillation fluid (Ecolite, ICN Biomedical Inc., Irvine CA) was added
to each vial. The radioactivity in the samples was counted in a Searle (Delta 300 model) liquid scintillation counter.

e) **Scanning electron microscopy**

The cells were prefixed in the Petri dishes, in complete medium for five minutes using 0.1% glutaraldehyde (Polysciences Inc., Warrington, PA). The glutaraldehyde-medium mixture was then replaced by 2% glutaraldehyde in 100 mM Na-cacodylate buffer (pH: 7.2) at room temperature for thirty minutes. Cells were postfixed in 17% osmium tetroxide for 30 minutes. After fixation the specimens were dehydrated for five minute periods through 50%, 70%, 90% and 100% ethanol and critical point dried with CO₂. The coverslips were removed and mounted using silver conducting paint. The samples were gold-sputter coated to a thickness of 50-70 nm, and examined with a JEOL ISM 35 CF, operated at an accelerating voltage of 25 kV. Selected areas were photographed for further examination.

**Results**

Data analysis

All dose-response data sets acquired from the present experiments exhibit three regions going from low to high doses: a) a "delayed response" region, with a lower threshold dose needed to observe any toxic effect; b) a "sensitive" region, that encompasses a range of doses where most of the toxic effect is exhibited; and, c) a "saturated"
response region where increasing the toxic dose does not produce any more toxic effect. Accordingly these curves were fitted by a two parameter sigmoid function given by the equation:

\[ Y = 100 \left(1-e^{-bx^n}\right) \]

where \( X \) = dose in \( \mu g/ml \) of protein and \( Y \) = response expressed as percentage of controls.

Transforming the previous equation into the following form:

\[ \ln(-\ln(1-Y/100)) = \ln b - n \ln X \]

permits the determination of the fitting parameters \( b \) and \( n \), using simple linear regression between \( \ln X \) and \( \ln(-\ln(1-Y/100)) \). The \( b \) and \( n \) parameters summarize the global information contained in the dose response curves and facilitate comparisons between them. The \( n \) parameter gives a measure of the sensitivity of the cell line to the substance tested (larger \( n \): larger sensitivity) while the \( b \) parameter gives an indication of the lower threshold dose ("activation dose") required to observe a toxic effect (larger \( b \): larger activation dose). Table 4 lists the \( b \) and \( n \) pairs for each dose response curve obtained. The ID50 dose (the dose that causes 50% inhibition of the measured function) was determined by solving the above equation for \( X \) with \( Y = 50 \). Table 4 also lists the ID50 doses for all experiments in this study. Comparison among the results of each experiment are performed based on the \( b \) and \( n \) values and on the ID50 values.

a) L 929 cells (murine fibroblasts)
The response of L 929 cells when exposed to *B. gingivalis* W 83 sonicated extracts is shown in Table 5. The doses used to challenge the cells varied between 100-250 µg/ml according to their protein concentration. The cell response is presented as percentage of controls. Each number represents the mean of triplicate cultures. Both evaluation methods (³H thymidine incorporation and cell counting) showed a similar trend. Figure 4 illustrates the relationship between the dose and the response of the cells when evaluated with both methods. With phase contrast microscopy, the cells seemed viable with no signs of morphological alterations in any of the concentrations tested. A decrease in cell density was evident in experimental cultures confirming the inhibition of cell proliferation shown quantitatively (Figs. 5A, 5B). SEM examination revealed no alterations of cell surface in any of the experimental cultures (Figs. 6A, and 6B).

The response to sonicated extracts from *B. gingivalis* ATCC 33277 is shown in Table 6. The cytotoxic effect of extracts from this bacterium was much stronger that the previous one, as expressed by decreased ³H thymidine incorporation and cell counts. Figure 7 illustrates the dose response curves with both evaluation methods. Morphological alterations were evident in light microscopy at concentrations above 200 µg/ml. Cells appeared shrunk, rounded in shape, detached from the substratum and some were floating in the medium.

The comparative toxicity of the two strains of *B. gingivalis* can be seen by comparing the doses that result in 50% decrease in numbers of the control cells. This dose is 209 µg/ml for the W83 strain and 91 µg/ml for the ATCC 33277 strain (Table 4).
b) Pulpal cells

Three pulpal fibroblast-like cell cultures (PL₂, PL₄, PL₅) were exposed to sonicated extracts of *B. gingivalis* W 83. The doses used to challenge the cells ranged between 100-250 μg/ml. The results of the cell response quantitated by 3H thymidine incorporation and cell counting are presented in Tables 7 and 8. Figures 8, 9 and 10 illustrate the dose response curves for each individual pulpal cell line with both evaluation methods. Figures 11 and 12 illustrate the response of all pulpal cell cultures as evaluated with either the thymidine incorporation method or with the cell counting method. All cell cultures exhibit a similar trend in the dose response curves independent of the method used. The individual cell responses to each dose were pooled to facilitate comparison between experiments. As can be seen from Table 7, all doses showed an inhibition of ³H thymidine incorporation for all three cell cultures. At the highest dose used (250 μg/ml), only 23.19±4.07% of the cells (pooled data) continue to incorporate thymidine (Table 7). At the same high dose the cell counts were 40.37±14.37% of control cultures (Table 8). Figure 13 is a graphic representation of this relationship.

At concentrations higher than 200 μg/ml, the pulpal cells looked remarkably different than their controls when evaluated with light microscopy (Figs.14 A,14B). Fewer cells were observed per surface area, while the medium contained various numbers of floating cells. Intercellular gap formation was accompanied by shriveling of the cells. The cells, in general, have lost their fibroblastic shape and were
irregular. These morphologic alterations were considered to be signs of major cell injury.

Two pulpal cell cultures (PL₆, PL₁₂) were exposed to sonicated extracts of *B. gingivalis* ATCC 33277. The doses used to challenge the cells were between 10-150 µg/ml. Cell response was evaluated by monitoring \(^3\)H thymidine incorporation, as shown in Table 9. Both cell lines show a dose dependent inhibition of \(^3\)H thymidine uptake. At the highest dose (150 µg/ml) less than 10% of the control cells incorporated thymidine. The dose response curves of both cell lines are plotted in Fig 15.

Comparison of pooled data from cell exposure to bacterial proteins revealed that pulp cells are by far more sensitive to *B. gingivalis* ATCC 33277 than to W 83 (Fig. 16). The concentration of proteins from the latter strain needed to elicit 50% inhibition of thymidine incorporation was 15 µg/ml (mean value) while the corresponding dose of *B. gingivalis* W 83 was 143 µg/ml (Table 4).

Light microscopic analysis confirmed the quantitative data. Severe alterations in cell morphology, similar to the ones described previously, were apparent at a protein concentration of 100 µg/ml. Intercellular gaps were bigger and certain areas of the attachment surface were devoid of cells presumably due to cell injury. These morphologic changes were observed as early as 6 hours after the addition of the bacterial sonicates to the medium. After 24 hours exposure to the highest protein dose (150 µg/ml) many cells were damaged beyond recognition (Figs.17A,17B)
Although there was an inhibition of $^3$H thymidine uptake by the cells when exposed to 10 µg/ml of B. gingivalis ATCC 33277 extracts no recognizable changes could be seen in the scanning electron microscopy evaluation (SEM). At 150 µg protein/ml, SEM observations confirmed that the cells were severely damaged (Figs. 18 A,B and 19 A,B).

Comparison of the dose response curves of L 929 cells and pulpal cells exposed to different doses of B. gingivalis (both strains) showed that pulpal cells are by far more sensitive than L 929 cells when exposed to bacterial sonicates (Fig.20, Fig.21). As an example B.gingivalis ATCC 33277 sonicate concentrations required to cause 50% inhibition of thymidine uptake were 114 µg/ml for the L 929 cells, and 15 µg/ml (mean value) for the pulpal cells (Table 4). The "activation" doses were also lower for the pulpal cells (Fig.20, Fig.21)

In summary, L 929 and pulpal cells showed a dose related cell growth inhibition when exposed to sonicates of both B. gingivalis strains. B. gingivalis ATCC 33277 sonicates appeared more toxic than B. gingivalis W 83 for all cell cultures. Comparison of the cell response to both bacterial sonicates suggested that pulpal cells were significantly more sensitive than L 929 cells.
PART 3:

The Moderating Effect of Dentin Membranes on the Cytotoxicity of Bacterial Proteins

The final part of the study focused on the role of dentin as a membrane through which transportation of bacterial byproducts can take place. An experimental model was developed to permit the study of the toxicity of bacterial extracts after diffusion through dentin.

Materials and Methods

a) The chamber

A modified Sykes Moore chamber was used for the experiment. The original chamber consists of five parts. Two stainless steel interlocking rings, two glass or plastic coverslips and one silicone rubber gasket. The various parts are shown in Fig.22. The stainless steel rings are interlocking by threads. This holds together the two coverslips which are separated by the silicon gasket. The bottom coverslip serves as the substratum for cell colonization. Through the silicon gasket the assembled chamber can be filled with cells in suspension, using a hypodermic needle. In the new modified chamber the top coverslip was replaced with a two piece assembly made of Lexan (Fig.23). In this assembly a dentin disk can be clamped in position between two silicone O-rings. The pulpal surface of the disk is in contact with the medium in the culture chamber and its coronal surface is exposed to the external environment (Fig.24). Thus access to the
cells in the culture chamber is possible through the dentin membrane. The surface area of the exposed dentin is controlled by the size of the O-rings (O Rings Incorporated, Los Angeles, CA). These O-rings are the same size as the ones used previously in the "split chamber device". Disinfection of this Lexan system was accomplished by submerging in alcohol, followed by rinsing in sterile distilled water, and overnight exposure to ultraviolet (UV) light. Sterilization of parts from the original Sykes Moore chamber was accomplished by autoclaving.

b) **The incorporation of dentin disks in the modified chamber**

The cells were cultured separately in Petri dishes as described in Section 2. At the time of the experiment the coverslip with the cells was transferred from the Petri dish to the Sykes Moore chamber. The gasket was fitted and the bottom part of the top assembly was placed in position and screwed tightly. Finally the dentin disk was positioned and stabilized after tightening the upper part of the apparatus. To ensure that the dentin disks to be used are permeable, the hydraulic conductance was determined using the apparatus described in Section 1. The dentin disks were stored in PBS and exposed to ultraviolet light overnight before each experiment.

c) **Cytotoxicity of bacterial proteins after diffusion through dentin membranes**

The monolayer culture in the lower part of the chamber was maintained in regular medium supplemented with antibiotics. The upper chamber (Fig.24) was filled with medium containing bacterial proteins
(experimental) or with medium alone (controls). Evaluation of the DNA synthesis of the challenged cells after direct or indirect exposure to bacterial proteins was performed with the labelled thymidine method. Comparison of the cell response values after direct exposure to bacterial proteins, with the cell response values after exposure to bacterial proteins through dentin barriers was the crucial factor in determining the attenuating role of dentin.

Three separate experiments were performed. Five chambers were used as experimental and two as controls in the first experiment. Six chambers were used in the second experiment. Pulpal cells (PL6 and PL12) between the fourth and fifth passage were used as target cells in the chambers. The experiments were performed with _B. gingivalis_ ATCC 33277, sonicated extracts as irritants. The bacterial sonicates used to indirectly challenge the pulpal cells were diluted in culture medium to a final concentration of 150 μg/ml and 300 μg/ml. A total of 11 disks were used. All disks were of the same thickness (0.500 ± 0.003 mm). The results were analyzed statistically using the t-test (Zar 1984).

**Results**

Results of bacterial protein diffusion through dentin disks are shown in Tables 10, 11 and 12. All experimental cell responses were statistically different than the controls (p<0.05). The relationship between thymidine uptake and bacterial protein dose (dose-response curve of fig.15) was used to estimate the amount of bacterial proteins that when diffusing through the dentin would produce the cytotoxic effect observed. These concentrations appear in the third column of Tables 10-
12. In the third experiment the response of the third chamber was considered atypical and was excluded as an outlier. Such high cellular response value was taken as a sign of contamination masking the toxic effect of the bacterial proteins. The cellular response in the three experiments suggest that less than 10% of the applied proteins are able to penetrate the dentin barrier.

It is of interest to use these concentrations in conjunction with Fick's Law of diffusion, to estimate the diffusion coefficient ($D$) of the bacterial proteins solution. Accordingly:

$$J = D_{\text{eff}} \Delta c / L$$  \hspace{1cm} (6)

We can solve for $D_{\text{eff}}$

$$D_{\text{eff}} = J L / \Delta c$$ \hspace{1cm} (7)

where:

$J$ = Diffusive flux

$L$ = Thickness of dentin disk

$\Delta c$ = Concentration difference between the two sites of dentin

The thickness of the dentin disks was 0.5mm and the concentration difference was 300 or 150 $\mu$g/ml.

The diffusive flux ($J$), can be estimated from the available experimental data as follows:

$$J = \text{Mass of solute} / (\text{Area x Time}) = (\text{Dose x Volume}) / (\text{Area x Time})$$
The available data are:

Dose = The dose provided in the third column of Tables 10, 11, 12 in μg/ml

Volume = Volume of the bottom chamber (0.55 ml)

Area = 0.1256 cm²

Time = 24 hours

Diffusion coefficients calculated from equation (7), for the three experiments are converted to free-diffusion coefficients ($D_{\text{free}}$) for the protein solutions used in the three experiments, using the formula:

$$D_{\text{free}} = \frac{D_{\text{eff}}}{\varepsilon}$$  \hspace{1cm} (8)

where $\varepsilon$ is the porosity of dentin ($\varepsilon = 7.89\%$ for surface treated dentin, as estimated by Pashley et al., 1978a)

Results from calculations of $D_{\text{free}}$ and $D_{\text{eff}}$ are presented in Table 13.
DISCUSSION

The present study investigated the various aspects of the toxicity of *B. gingivalis* extracts on pulpal cells and L 929 cells *in vitro*. After confirming that dentin is permeable to these extracts (Part 1), pulpal cells and L 929 cells were exposed directly to them (Part 2). To assess whether dentin allows diffusion of bacterial extracts pulpal cells were exposed to *B. gingivalis* in an environment intended to simulate the pulp chamber (Part 3). Evaluation of cell growth after direct and indirect challenging with bacterial extracts indicated that dentin restricts their transport. The findings of the present investigation are discussed below.

The permeability of the dentin specimens used in this study was calculated with the method developed by Reeder et al. (1978) and extensively applied by Pashley et al. (1978a,b, 1981a,b). This method was used because it defines dentinal surface area and thickness; thus, the parameter of cavity dimensions is controlled. Compared with other methods available for studying dentin permeability (Anderson and Ronning 1962; Smith and de Vicenzo 1968; Hume 1984) the present one, is considered more objective since it offers a standardized environment. The dentin slices used were cut as close to the pulp horn as possible, to avoid variation in sample morphology. Etching of the dentin specimens was performed in order to eliminate the smear layer and to decrease variability among samples.
To our knowledge, filtration of bacterial proteins through dentin has not been reported earlier. The results obtained in this study show that when a *B. gingivalis* ATCC 33277 sonicate solution (200 μg/ml of protein concentration) is forced at a pressure of 180 cm PBS the result is a significant reduction of dentin permeability (K' or Lp). This reduction in K' and Lp can be interpreted on the basis of Darcy's Law, the fundamental equation for fluid flow through porous media (Scheidegger 1960). Recall that K' = k / μ, and K' = Lp L. Since each disk served as its own control, changes in K' are identical to changes in Lp. In the following discussion we prefer the use of K' for comparisons among samples.

A change in K' can be attributed either to a change in the viscosity of the bacterial proteins (b.p) containing solution over that of pure PBS, to a change in the Darcy permeability of the dentin disk (i.e. to microstructural changes), or both. An estimation of the viscosity change of the PBS solution when b.p are added, can be made on the basis of Einstein's Law of viscosity for suspensions (Cohn and Edsall 1965).

\[ \frac{μ_{bp}}{μ_{PBS}} = 1 + 2.5 \varphi \]

where \( \varphi \) is the protein volume fraction of the b.p solution. We estimate \( \varphi \) to be around 10^{-4} from the concentration of the b.p containing solution (200 μg/ml) and assuming a typical protein density of 1.3 g/cm^3 (Cohn and Edsall 1965). This order of magnitude estimate, shows that the significant reduction in K' when b.p were used (Table 1) can be largely attributed to microstructural changes of dentin (changes in k) and that the effect of viscosity is negligible. These
microstructural changes are elucidated using a porous media model for the dentin such as a bundle of straight capillaries. The $K'$ of such a medium was derived from the Poiseuille-Hagen Law in the Introduction as:

$$K' = \pi r^4 N / 8 \mu A,$$

where $N$ is the number of tubules and $A$ is the surface area of the specimen. The variables in this relation are $r$ and $N$ since the rest of the quantities are constant for a given experiment. Protein retention can take place either on the surface of the disk or intratubularly. Accumulation of bacterial proteins on the surface of the disk can occlude the tubules partially or totally (reducing $N$ and/or $r$). Intratubular protein retention (reduction of $r$) involves the possible binding of the proteins to dentin hydroxyapatite, or lamina limitans.

The present results demonstrated a positive correlation between b.p. retention and reduction in $L_p$ (or $K'$), although the exact relationship is not simple, as is easily appreciated from the fact that some (three) disks showed a reduction in $L_p$ (or $K'$) without any measurable b.p. retention. Early obstruction of small tubule orifices by miniscule amounts of b.p. (below the detection limit of the spectrophotometric method used) may be responsible for these results. The inverse i.e. no reduction in $L_p$, with appreciable b.p. retention, was also observed in one disk. Deposition of b.p. on the frontal surface of the disk may account for this observation, although more extensive measurements are really required to arrive at definite conclusions. It should be kept in mind, that the whole experimentation is very technique sensitive. The collection of the protein solution, the spectrophotometric method of detecting protein concentrations, and the
possible denaturation of proteins within the time frame of the experiment, all could influence the experimental results.

Earlier studies have investigated the changes in dentin $L_p$ caused by bacterial treatment of dentin. Michelich et al. (1980) reported that the hydraulic conductance of the dentin disks when bacteria were filtrated was reduced by 50%. The number of microorganisms exiting the dentin surface represented only 0.2% of the entering ones. Pashley et al. (1982), also showed that filtration of bacteria decreases the $L_p$ of dentin, the decrease being associated with the concentration of microorganisms. It was speculated that lodging of bacteria in the tubule walls may account for the spontaneous elimination of hypersensitivity, often observed in clinical studies. In order to examine the correlation between $L_p$ reduction and the nature of the solute Pashley et al. (1982) used substances with standard molecular weights such as fibrinogen, gamma globulin, albumin and others. Reduction of $L_p$ similar to the one that was observed in this study, was shown with a 5% solution of gamma globulin with a molecular weight (MW) of 190,000. No speculations however can be made about the MW of the present experimental solution.

The decrease of the $K'$ was not irreversible in the time frame of the experiment as can be seen from Table 1, since most of the dentin specimens returned to their initial permeability. Using radioactivity labelled tetracycline as a tracer, Ciarlone et al. (1988) showed that the binding of tetracycline to dentin was also reversible. It was suggested that in vivo there are two steps in substance-dentin interactions. The first step is an initial binding reaction while the second step is a trapping inclusion. The trapping of any substance to
the dentinal tubules is susceptible to factors such as synthesis and mineralization of new dentin matrix that are not simulated in the present in vitro model. Under the conditions of the present experiment and based on the reversibility of $K'$, the intratubular interactions seem to have only a physical basis.

The possible effect of bacterial byproducts on the pulp was examined by exposing pulpal cells to B. gingivalis extracts. The use of pulpal cells has previously been reported in cytotoxicity studies (Miller et al., 1976; Munaco et al., 1978; Kawahara 1979; Das 1981; Feigal et al., 1985) but their use has not been extensive. In general, there is no conclusive information in the literature regarding the ideal culture and storage conditions for pulpal cells. Isolation of these cells from human or bovine dental pulp has been achieved using enzymatic cell separation (Miller et al., 1976; Levin et al., 1988). This technique, however, requires many dental pulps in order to produce an appreciable amount of cells; furthermore, it is still not clear which is the most appropriate enzyme to use. In addition, by pooling pulps from different donors, the possibility to distinctively isolate a cell line with different characteristics is eliminated.

In the present study, pulpal cells were grown from explants of human dental pulps. Each cell line represents a single pulp from a different donor. The need for only a single pulp organ for the start of each cell line was a significant advantage of the method. Growing cells from explants also facilitated fast isolation of many cell lines, thus permitting a comparison of their function. One intention of the present study was to search for the presence of different reactions between
various pulpal cell lines to bacterial byproducts, as such a phenomenon has been previously reported for gingival fibroblasts (Hassell et al., 1976; Larjava and Vitto, 1987). The pulpal cells obtained from the explants were fibroblast-like cells very similar in morphology to the ones described by Miller et al. (1976), Munaco et al. (1978), and Das (1981), but different than the ones shown by Kawahara et al. (1979). Differences in the isolation technique and in the medium used may account for this latter lack of similarity. The rationale behind the use of an established cell line (L 929) in a parallel experiment was to compare their response with the one of the pulpal cells and consider them as an alternative experimental cell. Previous experience and knowledge of a standardized cultural environment suitable for the L 929 cells, renders them more amenable for experimentation.

*B. gingivalis* extracts were used as an experimental irritant for the cells. This bacterium is one of the few species found under dental restorations (Mejare et al., 1979; Bergenholz et al., 1982). Many previous investigations have reported bacteria under dental restorations (Brannstrom and Nyborg 1971, 1973, 1974; Qvist 1975) but limitations in culture techniques for identification are responsible for the lack of more information on this subject. *B. gingivalis* has been among the most commonly used bacteria to challenge gingival fibroblasts and other cell lines in periodontal research with tissue cultures.

Extracts from two strains of *B. gingivalis* were chosen as irritants for the cells. *B. gingivalis* W 83 is a highly toxic strain capable of inducing rapidly spreading infections in mice and guinea pigs. Its virulence has been attributed to its high proteolytic and collagenolytic
activity (Sundqvist et al., 1987). Sonicated extracts from a W strain of *B. gingivalis* were shown to have the highest collagenolytic activity among many *Bacteroides* and *Actinobacillus* species (Robertson et al., 1982). Other investigators (Touw et al. 1982a,b; van Steenbergen et al. 1982; Grenier and Maynard 1985) also demonstrated the high cytotoxicity of *B. gingivalis* W 83 on Vero cells. In general the results from the present study agree with the previous information, since they do show an inhibition of DNA synthesis and cell growth and a significant morphological effect after 24 hours exposure of the pulp cells to *B. gingivalis* W 83 sonicated extracts. Since the nature of the cells are different, no detailed comparison can be made between the response of Vero cells to *B. gingivalis* W 83 sonicated extracts and the response of the pulpal cells and the L 929 cells found in this investigation. Previous studies do not specify the dosage of the bacterial irritant used to challenge the cell cultures and this lack of information makes comparison between studies impossible. In addition, the evaluation of the cell response by morphology, histology and trypan blue exclusion is subjective and prone to inter-examiner variability.

The cytotoxic effect to human gingival fibroblasts of *B. gingivalis* ATCC 33277 sonicates was previously demonstrated by Larjava and Vitto (1987) and Larjava et al. (1987). The dose that caused 50% inhibition of thymidine uptake (ID50) on twelve gingival fibroblast cell lines ranged from 8-60 μg/ml of protein concentration. In the present study the ID50 for L 929 cells was 114 μg/ml and for the pulpal cells ranged from 12-19 μg/ml on the two pulp cell lines studied. Under the conditions of the present experiment, L 929 cells are less sensitive
than gingival fibroblasts to extracts of *B. gingivalis* ATCC 33277. The sensitivity of the pulpal cells can be considered similar to that of gingival fibroblasts reported in previous studies. In the Larjava et al. (1987) study, the ID50 of the bacterial extracts for two of the gingival cell lines are similar with the ID50 for pulpal cells. However, for more definite conclusions larger numbers of pulpal cell lines have to be used so that possible variations in their response could be revealed.

Five individual pulpal cell lines were used in the present study. Three were exposed to *B. gingivalis* W 83, and two to *B. gingivalis* ATCC 33277. Although their response to the irritant was not the same, they showed a similar dose-dependent growth inhibition. *B. gingivalis* W 83 extracts were less toxic to the pulpal cells than *B. gingivalis* ATCC 33277. The same was true for the L 929 cells. Assuming that the bacteria were pure and based on the knowledge that the two strains have a very similar DNA homology (Coykendall et al. 1980), we suggest that the toxic factor is produced to a higher degree from the ATCC 33277 strain. A similar variation in cytotoxicity between different strains of the same microorganism has been reported earlier (Shenker et al. 1982; Boehringer et al. 1984). More research on the toxicity of extracts from bacteria known as pathogens to the pulp is certainly needed. Such information will permit for an even more comprehensive comparison between gingival fibroblasts and pulpal cells regarding their susceptibility to bacterial pathogens.

L 929 cells appeared less affected than pulpal cells (all cell lines) to both bacterial extracts, therefore they cannot substitute them
in such experimentation. These results agree with previous observations by Shenker et al. (1982), and by Boehringer et al. (1984) who reported higher sensitivity of human skin fibroblasts compared to L 929 cells. Collectively, this information would probably confirm our expectations; i.e., that L 929 cells as an immortal cell line should be less susceptible to an irritant. However, in biomaterial research many studies have shown the opposite (Spangberg 1969; Hanks 1981,1983; Al-Nazhan and Spangberg 1990). This suggests that the cell reaction to an irritant is critically dependent on the nature of the irritant.

The study of the susceptibility of different cell lines will be facilitated in the future when the nature of the cytotoxic factor present in bacterial sonicates will be known. Clarification of the irritation stimulus present in the bacterial sonicates is a subject of investigation. Heat stability, as a characteristic of the cytotoxic molecule in bacterial or plaque extracts has been reported by many investigators (van Steenbergen et al.1982; Larjava et al.1983; Larjava et al. 1987) who suggested that endotoxin is responsible for the extract's cytotoxicity supporting previous speculations by Aleo et al. (1974), and Daiyi et al. (1980). Dose dependent inhibition of cell growth from pure endotoxins from E. coli, B. gingivalis, and B. intermedius, was further shown by other investigators (Singer and Dutton, 1979; Olson et al., 1985; Layman and Diedrich, 1987; Layman and Landreneau, 1989). The single investigation using pulpal cells (Paterno et al. 1987) showed however, that the direct cytotoxic effect obtained from pure endotoxin was minimal compared with the one obtained from crude bacterial
preparations, since very high doses were needed to exert a comparable effect.

A heat stable cytotoxic factor extracted from cultured dental plaque has been previously described by Singer and Buckner (1980), who showed that it is butyric and propionico acids that are responsible for the cytotoxicity. Various harmful effects of butyric and propionico acid have been reported in the literature (for a review see Prasad and Sinha, 1976) and many investigators (Levine et al., 1973; Touw et al., 1982b; Grenier and Mayrand, 1985) have associated the cytotoxic effect of plaque or bacterial extracts with them. Other studies (Higerd et al., 1978; Boehringer et al., 1979; Shenker et al., 1982; Stevens et al., 1988) associated the growth inhibitory effect of different bacterial extracts with a high molecular weight, heat labile substance, most likely not endotoxin. Since the inhibitory factor is not purified and characterized, we have tentatively used terms such as "bacterial extracts" or "bacterial byproducts" to describe the crude preparation used as irritant. However the presence of a well defined population of proteins (Reed et al., 1982) and experimental evidence (Higerd et al., 1978; Shenker et al., 1982; Boeringer et al., 1984) that the cytotoxic activity is heat labile and of high molecular weight, justified the use of protein concentration as a means of comparison between experiments.

As an attempt to simulate the in vivo condition of diffusion of bacterial byproducts to the pulp, a pulp chamber-like system was manufactured. Pulpal cells, dentin and bacterial extracts diffusing through dentin are present in that system.
The modified Sykes Moore chamber fulfilled the need for a simple apparatus that would permit for the study of cells exposed to different substances through dentin. The original chamber provided an autoclavable container commonly used in tissue culture, that permits visualization of the cell cultures by phase contrast microscopy. The incorporation of the top Lexan assembly extended the range of applications of the original chamber. This part was designed so that a dentin disk could be positioned steadily on the top of the cultured cells and as close to them as possible. The experimental solution (here, bacterial sonicates) can be placed over the dentin and evaluation of the cell function gives indirect information about the degree of protection that dentin offers. The basic Sykes Moore chamber was preferred over other existing models basically because the cells can be cultured with small amounts of culture medium (0.55ml). This eliminates diffusion through the culture medium, as a limiting step in the kinetics of bacterial proteins-cell interactions. This was also considered to be a very important parameter by other investigators (Browne 1985; Hanks et al., 1989).

Pulpal cells were exposed to toxic bacterial sonicates passaged through dentin. The presence of the bacterial sonicates in culture medium at the top of the chamber creates a concentration gradient triggering diffusive transport. This study indicated that dentin presents a barrier to the diffusion of cytotoxic substances in vitro. In fact, in all three experiments, the cell response was equivalent to less than 10% of the direct protein dose used to challenge them. The high cellular response observed in one of the chambers in the third
experiment cannot be attributed to higher permeability of the particular dentin sample (due to exposed pulp horn in the dentin disk), since all disks had similar permeabilities. Thus, even very thin residual dentin (the thickness of dentin in the present experiments was only 0.5 mm) can offer protection to the pulpal cells. The present results are in accordance with results from previous studies showing that dentin significantly attenuates the potency of a chemical irritant passing through its structure. (Meryon 1984; Meryon and Jakeman 1986; Hume 1984; Hanks et al. 1988; 1989).

Although the role of dentin in absorbing toxic components of biomaterials has been studied in vitro by several investigators (Meryon, 1983, 1984; Hume, 1984, 1985; Meryon and Jakeman, 1986; Hanks et al., 1988, 1989), the mechanism regulating bacterial extract absorption is unknown. Meryon and Jakeman (1986), suggested that it is dentin's organic matrix that is mainly responsible for the absorption of the toxic components in biomaterial evaluation. It should be noted that in the "model cavity system" used by these investigators the cultured cells are separated from the dentin by a large amount of culture medium (4 ml) thus possibly contributing to false negative results. Hume (1984, 1985) experimented with attenuation of ZOE toxicity through dentin and focused on eugenol transport. Eugenol, however, presents many unique binding characteristics both in vivo and in vitro so this information cannot really be extrapolated to other biomaterials. Furthermore, in the model described by Hume (1984), the permeability of dentin was not by any means measured or standardized.
The "in vitro pulp chamber" (IVPC) developed by Hanks et al. (1988, 1989) simulated the in vivo conditions more closely than the previous available models. It was clearly pointed out by these investigators that the amount of culture medium separating cultured cells from the dentin was a very important experimental variable since it is capable of diluting the toxic components rendering them less harmful to the cells. Hanks et al. (1988, 1989) reported that dentin significantly reduces the chemical cytotoxicity of composites and this reduction was analogous to the thickness of the dentin. One common conclusion of these studies was that the presence of dentin in an in vitro model testing biocompatibility is essential. The results of the present study supported that concept by showing major differences in cell reactions, after direct and indirect exposure to the bacterial extracts.

The diffusion coefficient (D\text{eff}) computed from the present experimental data sets (Table 13) can be used to estimate how long it would take for the first bacterial protein molecule, to cross the dentin disk. Since the diffusion coefficient physically gives the mean square displacement of a molecule in the unit of time (Probstein 1989), ie:

\[
D_{\text{eff}} = \langle \Delta L \rangle^2 / t
\]  

(9)

we can estimate \( t = \langle \Delta L \rangle^2 / D_{\text{eff}} \)  

(10)

where \( \Delta L = 0.5 \text{mm} \)

and \( D_{\text{eff}} \) values are available from Table 13, for the three experiments.
It is estimated that it took six hours for the bacterial proteins to cross the dentin disk. This estimate suggests that the bacterial proteins solution tested has a "diffusion velocity" of $\frac{\Delta L}{t} = 2.31 \times 10^{-6}$ cm/sec or, 0.023 $\mu$m/sec. This can be contrasted to the mean "convective velocity" found in the first part of the present experiment ($v = \frac{Q}{A} = 0.509 \times 10^{-3}/(0.1256, 60)$ which was $6.754 \times 10^{-5}$ cm/sec. Thus, we see that an application of 180 cm H$_2$O pressure difference across dentin can make bacterial proteins transport approximately 29 times faster. These results are consistent with previously reported data from Merchant et al. (1977) indicating that the rate of iodide permeation through dentin by filtration was 32 times higher than that obtained by diffusion. Free-diffusion coefficients calculated from Eq. (8) are within the range of free-diffusion coefficients for proteins (Cohn and Edsall 1965). This lends confidence to the experimental design used and justifies the use of the laws of diffusion for this system.

Information regarding the time needed for the bacterial proteins to diffuse through dentin can be very useful for future experimentation. Experiments should permit a minimum time period for diffusion to reach a steady state condition. This minimum time can be evaluated from a preliminary calculation based on the diffusion coefficient of the toxic stimulant to be used and the geometry of the experimental device.

The newly designed chamber allows variation between experiments. By keeping all parameters constant and using disks of different thickness, the role of the dentin thickness can be investigated. The role of the surface area available for diffusion is also of major interest as Pashley et al. (1987) found regional variability in dentin
permeability. The present model standardizes the surface area with the O-rings. The amount of medium can be regulated also by using gaskets of different thickness in the Sykes-Moore chamber.

The modified Sykes-Moore chamber can have several applications in the future cytotoxicity research. The cytotoxicity of freshly mixed materials can be measured with numerous methods since the chamber permits for introduction of tracers, radioactive labels, etc. The chamber carries only 0.55 ml of culture medium which allows fast transportation of the toxic molecules to the cells and more accurate results. In addition, successful culturing of pulpal cells is feasible, which is another step closer to a true in vivo simulation. Use of the dentin disk as a cell attachment surface further adds to the model by allowing an even more direct measurement of the cytotoxic effect.

Perhaps the most significant application of the present chamber in the future will be in the detection of microleakage of restorative materials. A tooth crown with a restoration can be used instead of the dentin disk (Fig 25). Bacterial components applied over the tooth will most likely travel through leaking areas and dentin and reach the cells in culture. Evaluation of the cell function can be used to measure the additive toxic effect of the material and the leaking bacterial components. Distinction of the two cytotoxic factors is feasible had the bacterial irritant be added at a different time than the filling. Cell recovery after various exposure periods can also be measured after removal of the top chamber and renewal of medium. This chamber where all important parameters are controlled represents a refined method that
permits sensitive observation of cytotoxicity as well as microleakage of materials.

The proposed model establishes a sensitive \textit{in vitro} method to study bacterial byproduct diffusion through dentin. Future studies on the toxicity of different plaque bacteria to pulpal cells will be of major importance in the understanding of pulpal disease. The present model enriches the list of models available for biocompatibility evaluation with the distinct advantage that it can detect microleakage as well.
CONCLUSIONS

1. The permeability of dentin is significantly reduced during filtration of \textit{B. gingivalis} ATCC 33277 proteins \textit{in vitro}. This decrease in permeability was reversible under the conditions of the present experiment. Bacterial proteins are retained to various degrees in the dentin disks during filtration.

2. L 929 cells and pulpal cells from different donors, showed a dose related growth inhibition, after direct exposure to \textit{B. gingivalis} ATCC 33277 and W 83 proteins. Light and scanning electron microscopic analysis showed morphologic changes of the pulpal cells.

3. Pulpal cells were consistently more sensitive than L 929 cells to bacterial proteins. All pulpal cell lines used showed similar responses to bacterial proteins stimulation.

4. Dentin offered significant resistance to the diffusion of \textit{B. gingivalis} ATCC 33277 proteins \textit{in vitro}. Based on the Diffusion coefficients calculated from the experimental data, the "diffusion velocity" of the bacterial proteins solution through dentin was estimated to be 2.31x10^{-6} \text{ cm/sec.}
Figure 1.

Apparatus for dentin permeability measurements

Illustration of the apparatus used to measure the permeability of dentin in vitro. The movement of the air bubble represents the rate of fluid filtering across dentin. Adjustment of the microsyringe permits measurements of fluid flow. The various parts of the "split chamber" are shown separately in the right side of the diagram.
Figure 2.

Dentin hydraulic conductance with PBS versus dentin hydraulic conductance with bacterial extracts

Relationship between hydraulic conductance of dentin disks when PBS was used as a filtrate ($L_{p1}$), and hydraulic conductance of the disks when bacterial extracts were used as a filtrate ($L_{p2}$). Dotted line represents line of perfect agreement.
Figure 3.

Dentin hydraulic conductance before and after bacterial extracts filtration

Relationship between hydraulic conductances of the dentin disks before and after bacterial extracts' filtration through them ($L_{p1}$ versus $L_{p3}$)
Figure 4.

Effect of \textit{Bacteroides gingivalis} W 83 extracts on L 929 cells

Effect of \textit{Bacteroides gingivalis} W 83 extracts on L 929 cells, after 24 hours of incubation. \textsuperscript{3}H thymidine incorporation and cell counting were used to evaluate cell growth. Mean ± SEM of three replicate samples for each dose are shown. Both evaluation methods show an inhibition of cell proliferation with the challenging doses.
Figures 5A.

Phase contrast micrographs of L 929 cells exposed to *Bacteroides gingivalis* W 83

Phase contrast micrograph of L 929 cells exposed for 24 hours to *Bacteroides gingivalis* W 83 (250 µg/ml of protein concentration). The cells appear normal in shape, their only difference from the controls being the density of the monolayer (x42).

Figure 5B.

Phase contrast micrograph of non challenged L 929 cells (controls)

Phase contrast micrograph of non challenged L 929 cells (controls). Many mitotic figures appear in the field, indicating proliferative activity.(x42).


Figure 6A.

Scanning electron micrograph of L 929 cells exposed to *Bacteroides gingivalis* W 83

Scanning electron micrograph of L 929 cells exposed for 24 hours to *Bacteroides gingivalis* W 83 (250 µg/ml of protein concentration). The cells are spindle shaped with microvilli extruding from their surface (x500).

Figure 6B.

Scanning electron micrograph of non challenged L 929 cells (controls)

Scanning electron micrograph of non challenged L 929 cells (controls). Cells appear well attached with numerous blebs in their surface. Large number of mitoses are seen (x500).
Figure 7.

Effect of *Bacteroides gingivalis* ATCC 33277 on L 929 cells

Effect of *Bacteroides gingivalis* ATCC 33277 on L 929 cells, after 24 hours of incubation. Mean ± SEM of three replicate samples are shown. Inhibition of cell growth is shown with both evaluation methods.
Figure 8.

Effect of *Bacteroides gingivalis* W 83 extracts on pulpal cells (PL2)

Effect of *Bacteroides gingivalis* W 83 extracts on pulpal cells (PL2) after 24 hours of incubation. Inhibition of cell growth is shown with both evaluation methods.
Effect of *Bacteroides gingivalis* W 83 extracts on pulpal cells (PL₄)

Effect of *Bacteroides gingivalis* W 83 extracts on pulpal cells (PL₄) after 24 hours of incubation. Inhibition of cell growth is shown with both evaluation methods.
B. gingivalis W 83
Pulpal cells PL

% of controls

o o o o o (³H)-thymidine
□ □ □ □ □ cell counts

Dose (µg/ml)
Figure 10.

Effect of *Bacteroides gingivalis* W 83 extracts on pulpal cells (PL$_3$) after 24 hours of incubation. Inhibition of cell growth is shown with both evaluation methods.
Figure 11.

Effect of *Bacteroides gingivalis* W 83 extracts on three pulpal cell lines (PL₂, PL₄, PL₅), evaluated with thymidine incorporation.

Effect of *Bacteroides gingivalis* W 83 extracts on three pulpal cell lines (PL₂, PL₄, PL₅). The cell response was evaluated with thymidine incorporation. Mean ± SEM of three replicate cultures for each dose are shown. The three cell lines show a similar dose dependent inhibition of thymidine uptake. Note differences in the "activation" doses.
Figure 12.

Effect of *Bacteroides gingivalis* W 83 extracts on three pulpal cell lines (PL₂, PL₄, PL₅), evaluated with cell counting.

Effect of *Bacteroides gingivalis* W 83 extracts on three pulpal cell lines (PL₂, PL₄, PL₅). The cell response was evaluated by cell counting. Mean ± SEM of three replicate cultures for each dose are shown. The three cell lines show a similar decrease in cell proliferation with the challenging doses.
Comparison of the dose-response curves from all pulpal cell lines. Both evaluation methods show the same trend.
B. gingivalis W 83
Pulpal cells
(pooled data)

○○○○○ (³H)-thymidine
□□□□ cell counts
Figure 14A.

Phase contrast micrograph of pulpal cells exposed to *Bacteroides gingivalis* W 83

Phase contrast micrograph illustrating pulpal cells exposed for 24 hours to *Bacteroides gingivalis* W 83 (250μg/ml of protein concentration). Some cells are rounded and detached from the substratum, while others have lost their fibroblastic shape. Fewer cells appear in the field, compared to their controls (x42)

Figure 14B.

Phase contrast micrograph of non treated pulp cells (controls)

Phase contrast micrograph of non treated pulp cells (controls). The cells appear fibroblastic in nature, forming bundles in certain areas (center). Mitotic figures are present (x42).
Figure 15.

Effect of *Bacteroides gingivalis* ATCC 33277 extracts on pulpal cells

Effect of *Bacteroides gingivalis* ATCC 33277 extracts on pulpal cells after 24 hours of incubation. $^3$H thymidine was used as an evaluation method. Mean ± SEM of three replicate samples for each dose are shown. All doses show an inhibition of thymidine incorporation.
B. gingivalis ATCC 33277
Pulpal cells
(\(^3\)H) - thymidine

○○○○○ PL₉
□□□□□ PL₁₂

% of controls

\text{dose (ug/ml)}
Figure 16.

Comparison between the cytotoxicity of the two strains of *Bacteroides gingivalis* extracts, on pulpal cells

Comparison between the $^3$H thymidine inhibitory effect elicited by the two strains of *Bacteroides gingivalis* extracts, on pulpal cells. *Bacteroides gingivalis* ATCC 33277 appears to have a more profound effect.
Pooled data
Pulpal cells
($^3$H)–thymidine
B. gingivalis

○○○○○ W 83
□□□□□ ATCC 33277
Figure 17A.
Phase contrast micrograph of pulpal cells exposed to *Bacteroides gingivalis* ATCC 33277

Phase contrast micrograph illustrating pulp cells exposed for 24 hours to *Bacteroides gingivalis* ATCC 33277 (150 µg/ml of protein concentration). Certain areas show floating cells, while the rest of the field is occupied by cells damaged to different degrees (x42).

Figure 17B.
Phase contrast micrograph of non challenged pulpal cells (controls)

Phase contrast micrograph of non challenged pulpal cells (controls). The culture appears confluent with fibroblastic type cells. Formation of bundles is evident (x42).
Figure 18A.
Scanning electron micrograph of pulpal cells exposed to *Bacteroides gingivalis* ATCC 33277 extracts (x100)

Scanning electron micrograph of pulpal cells exposed for 24 hours to *Bacteroides gingivalis* ATCC 33277 (150 µg/ml of protein concentration) extracts. The cells are not well spread on the substratum with signs of cracks in their surface. Many areas appear devoid of cells, with lots of cell fragments present (x100).

Figure 18B.
Scanning electron micrograph of non-challenged pulpal cells (x100)

Scanning electron micrograph of non-challenged pulpal cells (controls). The field is occupied by well attached cells exhibiting numerous projections for attachment (x100).
Figure 19A.

Scanning electron micrograph of pulpal cells exposed to *Bacteroides gingivalis* ATCC 33277 (x500)

Scanning electron micrograph of pulpal cells exposed for 24 hours to *Bacteroides gingivalis* ATCC 33277 (150 μg/ml of protein concentration). Note irregular shape of the cells as well as numerous cracks in their surface (x500).

Figure 19B.

Scanning electron micrograph of control pulpal cells

Scanning electron micrograph of control pulpal cells. They appear flattened and well attached to the substratum. Their surfaces are smooth with no cracks apparent (x500).
Figure 20.

Comparison of the cytotoxicity of *Bacteroides gingivalis* W 83 on L 929 and pulpal cells

Comparison of the $^3$H thymidine inhibitory effect of *Bacteroides gingivalis* W 83 on L 929 and pulpal cells. Pulpal cells appear more sensitive at all doses.
Figure 21.

Comparison of the cytotoxicity of *Bacteroides gingivalis* ATCC 33277 on L 929 and pulpal cells

Comparison of the $^3$H thymidine inhibitory effect of *Bacteroides gingivalis* ATCC 33277 on L 929 and pulpal cells. Pulpal cells are exhibiting higher sensitivity than L 929 cells in all doses used.
B. gingivalis ATCC 33277
(H)-thymidine

○○○○○ L 929 cells
□□□□□ Pulpal cells (pooled data)
Figure 22.

The Sykes-Moore chamber

Assembled Sykes-Moore chamber in external and cross section view. 1=Bottom part, 2=Lower coverslip, 3=silicon gasket, 4=upper coverslip and 5=Top. The sequence of assembly is according to the numbering in the figure.
Figure 23.

The modified Sykes-Moore chamber

Modification of Sykes-Moore chamber. The top assembly replaced the upper coverslip.
Figure 24.
The modified Sykes-Moore chamber in external and cross section view

Modified Sykes-Moore chamber in external and cross section view. 1=Bottom part of the assembly, 2=O-ring, 3=Dentin disk, and 4=Top part of the assembly.
Figure 25.

A new microleakage model

Use of the modified chamber as a microleakage model. A restored tooth is replacing the dentin disk.
### Table 1
Hydraulic Conductance of Dentin Disk

<table>
<thead>
<tr>
<th>Disk</th>
<th>Thickness*</th>
<th>( l_{p1}^{**} \times 10^{-2} )</th>
<th>( K_1/\mu_1^{***} \times 10^{-3} )</th>
<th>( l_{p2}^{**} \times 10^{-2} )</th>
<th>( K_2/\mu_2^{***} \times 10^{-3} )</th>
<th>( l_{p3}^{**} \times 10^{-2} )</th>
<th>( K_3/\mu_3^{***} \times 10^{-3} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.36</td>
<td>1.33</td>
<td>0.48</td>
<td>1.32</td>
<td>0.47</td>
<td>2.12</td>
<td>0.76</td>
</tr>
<tr>
<td>2</td>
<td>0.37</td>
<td>4.42</td>
<td>1.63</td>
<td>2.21</td>
<td>0.81</td>
<td>***</td>
<td>****</td>
</tr>
<tr>
<td>3</td>
<td>0.38</td>
<td>5.88</td>
<td>2.23</td>
<td>2.61</td>
<td>0.96</td>
<td>5.30</td>
<td>2.01</td>
</tr>
<tr>
<td>4</td>
<td>0.44</td>
<td>5.22</td>
<td>2.51</td>
<td>2.08</td>
<td>0.91</td>
<td>4.51</td>
<td>1.95</td>
</tr>
<tr>
<td>5</td>
<td>0.30</td>
<td>2.43</td>
<td>0.73</td>
<td>1.46</td>
<td>0.44</td>
<td>2.52</td>
<td>0.75</td>
</tr>
<tr>
<td>6</td>
<td>0.41</td>
<td>1.99</td>
<td>0.81</td>
<td>1.45</td>
<td>0.59</td>
<td>2.65</td>
<td>1.08</td>
</tr>
<tr>
<td>7</td>
<td>0.55</td>
<td>1.76</td>
<td>0.96</td>
<td>1.76</td>
<td>0.97</td>
<td>2.65</td>
<td>1.45</td>
</tr>
<tr>
<td>8</td>
<td>0.31</td>
<td>5.17</td>
<td>1.60</td>
<td>2.88</td>
<td>0.83</td>
<td>5.32</td>
<td>1.64</td>
</tr>
<tr>
<td>9</td>
<td>0.31</td>
<td>4.24</td>
<td>1.31</td>
<td>3.40</td>
<td>1.05</td>
<td>6.10</td>
<td>1.89</td>
</tr>
<tr>
<td>10</td>
<td>0.36</td>
<td>2.49</td>
<td>0.89</td>
<td>1.95</td>
<td>0.69</td>
<td>2.51</td>
<td>0.90</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.36</td>
<td>3.52</td>
<td>1.40</td>
<td>2.09</td>
<td>0.77</td>
<td>3.74</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>0.08</td>
<td>1.75</td>
<td>0.67</td>
<td>0.65</td>
<td>0.21</td>
<td>1.54</td>
<td>0.52</td>
<td></td>
</tr>
</tbody>
</table>

*Thickness in mm

**\( l \) = hydraulic conductance in \( \mu l \) (multiply by 1.69 \times 10^{-5} \text{ cm}^2/\text{dyn. sec} \) to convert in C.G.S.

***\( \psi \) = permeability constant in \( \mu l \) (multiply by 1.69 \times 10^{-5} \text{ cm} \) to convert in C.G.S.

****Disk 2 was discarded after accidental damage
Table 2
Bacterial Proteins Filtration Through Dentin Disks
(B. gingivalis ATCC 33277 proteins 200 µg/ml were used as filtrate)

<table>
<thead>
<tr>
<th>Disk</th>
<th>Thickness (in mm)</th>
<th>B.P. Conc Outwards (in µg/ml)</th>
<th>% Retention in B.P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.36</td>
<td>49</td>
<td>75.5%</td>
</tr>
<tr>
<td>2</td>
<td>0.37</td>
<td>137</td>
<td>31.5%</td>
</tr>
<tr>
<td>3</td>
<td>0.38</td>
<td>131</td>
<td>34.5%</td>
</tr>
<tr>
<td>4</td>
<td>0.44</td>
<td>116</td>
<td>42.0%</td>
</tr>
<tr>
<td>5</td>
<td>0.30</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0.41</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0.55</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0.31</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0.31</td>
<td>155</td>
<td>22.5%</td>
</tr>
<tr>
<td>10</td>
<td>0.36</td>
<td>128</td>
<td>36.0%</td>
</tr>
</tbody>
</table>
Table 3
Change in Hydraulic Conductance in Correlation with Bacterial Proteins Retention

<table>
<thead>
<tr>
<th>Disk</th>
<th>Thickness (in mm)</th>
<th>% Reduction in Lp</th>
<th>% B.P. Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.36</td>
<td>0</td>
<td>75.5%</td>
</tr>
<tr>
<td>2</td>
<td>0.37</td>
<td>50.0%</td>
<td>31.5%</td>
</tr>
<tr>
<td>3</td>
<td>0.38</td>
<td>55.5%</td>
<td>34.5%</td>
</tr>
<tr>
<td>4</td>
<td>0.44</td>
<td>63.6%</td>
<td>42.0%</td>
</tr>
<tr>
<td>5</td>
<td>0.30</td>
<td>39.6%</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0.41</td>
<td>26.8%</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0.55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0.31</td>
<td>48%</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0.31</td>
<td>19.8%</td>
<td>22.5%</td>
</tr>
<tr>
<td>10</td>
<td>0.36</td>
<td>13.8%</td>
<td>36.0%</td>
</tr>
<tr>
<td>Dose Response Curves</td>
<td>b (Indication of Activation Dose)</td>
<td>n (Indication of Sensitivity)</td>
<td>ID50 in μg/ml</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>-----------------------------------</td>
<td>-------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>L929 cells exposed to B. ging. W83</td>
<td>26325.5</td>
<td>1.84804</td>
<td>309</td>
</tr>
<tr>
<td>(thymidine incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L929 cells exposed to B. ging. W83</td>
<td>1078.9</td>
<td>1.37567</td>
<td>209</td>
</tr>
<tr>
<td>(cell counting)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L929 cells exposed to B. ging. ATCC 33277</td>
<td>213765.6</td>
<td>2.66703</td>
<td>114</td>
</tr>
<tr>
<td>(thymidine incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L929 cells exposed to B. ging. ATCC 33277</td>
<td>270545.3</td>
<td>2.85358</td>
<td>91</td>
</tr>
<tr>
<td>(cell counting)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL2 exposed to B. ging. W83</td>
<td>1335112</td>
<td>2.81648</td>
<td>170</td>
</tr>
<tr>
<td>(thymidine incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL4 exposed to B. ging. W83</td>
<td>6737.5</td>
<td>1.88107</td>
<td>132</td>
</tr>
<tr>
<td>(thymidine incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL5 exposed to B. ging. W83</td>
<td>347.5</td>
<td>1.27892</td>
<td>129</td>
</tr>
<tr>
<td>(thymidine incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL2 exposed to B. ging. W83</td>
<td>57.6</td>
<td>0.756237</td>
<td>345</td>
</tr>
<tr>
<td>(cell counting)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL4 exposed to B. ging. W83</td>
<td>48.1</td>
<td>0.813740</td>
<td>183</td>
</tr>
<tr>
<td>(cell counting)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL5 exposed to B. ging. W83</td>
<td>113871</td>
<td>2.35176</td>
<td>165</td>
</tr>
<tr>
<td>(cell counting)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL6 exposed to B. ging. ATCC 33277</td>
<td>9.6</td>
<td>0.895288</td>
<td>19</td>
</tr>
<tr>
<td>(thymidine incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL12 exposed to B. ging. ATCC 33277</td>
<td>11.5</td>
<td>1.14465</td>
<td>12</td>
</tr>
<tr>
<td>(thymidine incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5

Effect of *B. gingivalis* W 83 Sonicated Extracts on L 929 Cells
(Numbers Represent % of Controls ± SEM)

<table>
<thead>
<tr>
<th>Bacterial Proteins (in µg/ml)</th>
<th>$^3$H Thymidine Incorporation</th>
<th>Cell Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>93.95 ± 5.52</td>
<td>79.03 ± 12.96</td>
</tr>
<tr>
<td>150</td>
<td>97.25 ± 3.37</td>
<td>74.66 ± 6.58</td>
</tr>
<tr>
<td>200</td>
<td>75.24 ± 13.38</td>
<td>50.93 ± 8.78</td>
</tr>
<tr>
<td>250</td>
<td>56.98 ± 0.51</td>
<td>38.68 ± 4.23</td>
</tr>
</tbody>
</table>

Table 6

Effect of *B. gingivalis* ATCC 33277 Sonicated Extracts on L 929 Cells
(Numbers Represent % of Controls ± SEM)

<table>
<thead>
<tr>
<th>Bacterial Proteins (in µg/ml)</th>
<th>$^3$H Thymidine Incorporation</th>
<th>Cell Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>63.04 ± 5.79</td>
<td>41.40 ± 6.85</td>
</tr>
<tr>
<td>200</td>
<td>14.38 ± 0.24</td>
<td>7.14 ± 0.94</td>
</tr>
<tr>
<td>250</td>
<td>8.38 ± 0.93</td>
<td>3.89 ± 0.95</td>
</tr>
</tbody>
</table>
Table 7

Effect of *B. gingivalis* W 83 Sonicated Extracts on Pulpal Cell Lines as Judged by $^3$H Thymidine Incorporation

(Numbers Represent % of Controls ± SEM)

<table>
<thead>
<tr>
<th>Bacterial Proteins in μg/ml</th>
<th>PL₂</th>
<th>PL₄</th>
<th>PL₅</th>
<th>Pooled Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>89.33 ± 9.57</td>
<td>66.56 ± 6.03</td>
<td>57.58 ± 0.29</td>
<td>75.04 ± 15.21</td>
</tr>
<tr>
<td>150</td>
<td>55.10 ± 7.41</td>
<td>51.09 ± 4.36</td>
<td>48.80 ± 1.01</td>
<td>54.76 ± 7.19</td>
</tr>
<tr>
<td>200</td>
<td>32.47 ± 0.68</td>
<td>23.00 ± 2.86</td>
<td>33.16 ± 1.14</td>
<td>29.40 ± 6.13</td>
</tr>
<tr>
<td>250</td>
<td>24.64 ± 4.92</td>
<td>20.77 ± 5.84</td>
<td>24.16 ± 2.62</td>
<td>23.19 ± 4.07</td>
</tr>
</tbody>
</table>
Table 8

Effect of *B. gingivalis* W 83 Sonicated Extracts on Pulpal Cell Lines as Judged by Cell Counting
(Numbers Represent % of Controls ± SEM)

<table>
<thead>
<tr>
<th>Bacterial Proteins in µg/ml</th>
<th>PL₂</th>
<th>PL₄</th>
<th>PL₅</th>
<th>Pooled Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>80.13 ± 14.31</td>
<td>66.04 ± 4.81</td>
<td>83.66 ± 11.25</td>
<td>76.61 ± 12.40</td>
</tr>
<tr>
<td>150</td>
<td>73.93 ± 13.08</td>
<td>61.00 ± 1.49</td>
<td>72.56 ± 1.55</td>
<td>69.65 ± 8.06</td>
</tr>
<tr>
<td>200</td>
<td>66.22 ± 4.12</td>
<td>46.58 ± 0.40</td>
<td>28.12 ± 3.68</td>
<td>47.08 ± 19.32</td>
</tr>
<tr>
<td>250</td>
<td>56.57 ± 0.23</td>
<td>40.08 ± 1.06</td>
<td>24.47 ± 1.06</td>
<td>40.37 ± 14.37</td>
</tr>
</tbody>
</table>

Table 9

Effects of *B. gingivalis* ATCC 33277 Sonicated Extracts on Pulpal Cell Lines as Judged by ³H Thymidine Incorporation
(Numbers Represent % of Controls ± SEM)

<table>
<thead>
<tr>
<th>Bacterial Proteins (in µg/ml)</th>
<th>PL₆</th>
<th>PL₁₂</th>
<th>Pooled Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>65.11 ± 6.88</td>
<td>50.74 ± 9.70</td>
<td>59.36 ± 10.45</td>
</tr>
<tr>
<td>50</td>
<td>38.11 ± 8.49</td>
<td>15.42 ± 3.40</td>
<td>26.76 ± 14.12</td>
</tr>
<tr>
<td>100</td>
<td>16.83 ± 1.03</td>
<td>9.69 ± 4.12</td>
<td>13.26 ± 4.80</td>
</tr>
<tr>
<td>150</td>
<td>6.70 ± 0.18</td>
<td>2.62 ± 0.70</td>
<td>4.28 ± 2.33</td>
</tr>
</tbody>
</table>
Table 10

Thymidine Uptake of Pulpal Cells (PL6 Fourth Passage)
When Challenged through dentin with *B. gingivalis* ATCC 33277 (300 μg/ml)

<table>
<thead>
<tr>
<th>Chamber</th>
<th>$^3$H Thymidine Uptake (% of Controls)</th>
<th>Corresponding Dose (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62.58%</td>
<td>12.77</td>
</tr>
<tr>
<td>2</td>
<td>63.51%</td>
<td>12.42</td>
</tr>
<tr>
<td>3</td>
<td>51.02%</td>
<td>18.26</td>
</tr>
<tr>
<td>4</td>
<td>71.45%</td>
<td>9.73</td>
</tr>
<tr>
<td>5</td>
<td>86.64%</td>
<td>5.73</td>
</tr>
<tr>
<td>6</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

Mean = $11.78 \pm 4.50 \mu g/ml$
$= 3.92\%$ of the initial dose
### Table 11

Thymidine Uptake of Pulpal Cells (PL₆ Fifth Passage) when challenged with *B. gingivalis* ATCC 33277 (150µg/ml) Through Dentin

<table>
<thead>
<tr>
<th>Chamber</th>
<th>$^3$H Thymidine Uptake (% of Controls)</th>
<th>Corresponding Dose (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>91.90%</td>
<td>4.48</td>
</tr>
<tr>
<td>2</td>
<td>82.62%</td>
<td>6.71</td>
</tr>
<tr>
<td>3</td>
<td>69.15%</td>
<td>10.46</td>
</tr>
<tr>
<td>4</td>
<td>71.25%</td>
<td>10.87</td>
</tr>
<tr>
<td>5</td>
<td>67.89%</td>
<td>9.80</td>
</tr>
<tr>
<td>6</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

Mean = $8.46 \pm 2.76$ µg/ml

= 5.66% of the initial dose
Table 12

Thymidine Uptake of Pulpal Cells (PL₁₂ Fourth Passage) when challenged with *B. gingivalis* ATCC 33277 (150 μg/ml) Through Dentin

<table>
<thead>
<tr>
<th>Chamber</th>
<th>(^3)H Thymidine Uptake (% of Controls)</th>
<th>Corresponding Dose (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75.70%</td>
<td>6.28</td>
</tr>
<tr>
<td>2</td>
<td>68.19%</td>
<td>7.56</td>
</tr>
<tr>
<td>3</td>
<td>11.46%</td>
<td>53.56</td>
</tr>
<tr>
<td>4</td>
<td>85.50%</td>
<td>4.79</td>
</tr>
<tr>
<td>5</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

Mean (excluding 3rd chamber's response) = \(6.21 \pm 1.38 \mu g/ml\) = 4.14% of the initial dose
Table 13

Inferred Bacterial Proteins Diffusion Coefficients Through Dentin

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$J$ (in g/cm²·sec)</th>
<th>$D_{\text{free}} = \frac{D_{\text{eff}}}{e}$ (in cm²/sec)</th>
<th>$D_{\text{eff}}$ (in cm²/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>$5.9704\cdot10^{-10}$</td>
<td>$1.261\cdot10^{-6}$</td>
<td>$9.95\cdot10^{-8}$</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>$4.2877\cdot10^{-10}$</td>
<td>$1.811\cdot10^{-6}$</td>
<td>$1.43\cdot10^{-7}$</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>$3.1474\cdot10^{-10}$</td>
<td>$1.330\cdot10^{-6}$</td>
<td>$1.04\cdot10^{-7}$</td>
</tr>
</tbody>
</table>

Mean $D_{\text{eff}} = 1.1577\cdot10^{-7}$ cm²/sec ± 2.365·10⁻⁵
REFERENCES


Harper W.E. The character of the adaptation of the amalgam to the walls of cavities by present methods of instrumentation and the use of the best known alloys as indicated by the air pressure test. Dent. Rev. 26:1179-1198, 1912.


Kidd E. Microleakage: a review. J. Dent. 4:199-206, 1976


